STUDY OF SHEDDING PATTERNS OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN FECES, MILK, AND COLOSTRUM OF DAIRY COWS AND THE DEVELOPMENT OF NOVEL EARLY DETECTION METHODS FOR JOHNE'S DISEASE

BY

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ABSTRACT

The focus of this research was to improve *Mycobacterium avium* subsp. *paratuberculosis* (MAP) control and management programs through increased understanding of the diagnostic detection ability of common pathogen and antibody assays over seasons and lactation stages, as well as through the novel use of MAP-specific proteins and a cell-transport media for early diagnostic potential.

Solid and broth cultures, real-time PCR (qPCR), and ELISA diagnostic tests were used to assess a 12-month period of fecal, milk, and colostrum sampling from 51 MAP-infectious dairy cows and 52 consistently test-negative cows from four farms in New Brunswick and three farms in Prince Edward Island, Canada.

For all fecal, milk, and colostrum samples, improved detection ability for MAP was observed with qPCR over culture methods. The bacteria was four times more likely to be detected in feces than in milk with the same testing method in both sample types, but MAP was two times more likely to be detected in feces than in colostrum with qPCR testing. Milk ELISA detected antibodies three out of ten sampling times, but detection ability improved as fecal shedding or host age increased.

Seasonal effects were observed for qPCR and milk ELISA results. Higher detection ability occurred in winter and spring for fecal qPCR, in summer for milk qPCR, and in winter for milk ELISA. Summer held the best agreement between milk and fecal samples collected within the same month. In addition, milk ELISA showed better detection levels in late lactation animals.

MAP virulence proteins, including protein tyrosine phosphatase A (PtpA) and protein kinase G (PknG), were evaluated for use in a novel ELISA antigen-coating in milk and serum samples and as antigens for the interferon-gamma (IFN-γ) assay. In addition to the previous samples, extra serum and colostrum samples from cows from the same herds, and serum samples from cows from a test-negative herd were assessed. Improved detection of PtpA was seen in milk and PknG in colostrum, but results were inconsistent and variable. Production of IFN-γ was detected in interleukin-12 (IL-12) p40-potentiated PtpA and PknG whole blood samples. However, small sample size, low protein availability, and inconsistent results in the infected (two cows) and non-infected (two cows) animals biased the apparent benefit of these virulence proteins.

In addition, a novel cell transport media was assessed for improving the utilization of the IFN-γ assay. Whole blood samples from ten healthy cows were each divided into test and control samples, stimulated with the mitogens pokeweed and concanavalinA (ConA), as well as IL-12 p40, on days 1, 5, and 8 post-sampling. Functional immune response was assessed through IFN-γ production (ELISA) and mononuclear cell viability (propidium-iodide flow cytometry). Addition of the transport media improved ConA-dependent IFN-γ secretion and improved mononuclear cell viability up to eight days post-collection.

Improving our knowledge of factors affecting the interpretation of diagnostic assays could benefit Johne's disease management programs through more effective diagnostic strategies and increased producer participation. Results of this research support the use of qPCR in a Johne's herd control program, particularly to help minimize MAP exposure to calves. Furthermore, a simple method to extend white blood

cell viability *in vitro* can enhance the practicality of the IFN-γ test as another means of detecting MAP exposure and future risk of MAP infection and transmission within a herd.

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There may have been a mountain-range of laboratory work, but there were equally large statistical and epidemiological mountains to challenge, but the climb would not have been possible without the expertise of Drs. Henrik Stryhn, Javier Sanchez, Ian Dohoo, Tim Burnley, and Raphael Vanderstichel! I am forever grateful to have had the privilege to learn and work with such outstanding geniuses. Thank you so much for your patience, time, and help – and for making it look easy in the end. AVC is a better place because of all of you, and I know my gratitude is echoed by every graduate student that walks through its doors.

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DEDICATION

This thesis is dedicated, with so much gratitude, to someone who has never lost faith in me and has always been a wealth of encouragement and love.
This is for you, Mom.

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LIST OF ABBREVIATIONS

AVC	Atlantic Veterinary College
BHI	brain heart infusion
CI	confidence interval
ConA	concanavalin A
Ct	cycle threshold
CFU	colony forming unit
DIM	days in milk
ELISA	enzyme linked immunosorbent assay
g	gram
g	centrifugal force
HEYM	Herrold's egg yolk medium
HPC	hexadecylpyridinium chloride monohydrate
HRP	horse radish peroxidase
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MHC	major histocompatibility complex
ml	milliliter
MQM	Maritime Quality Milk
NK	natural killer cells
nm	nanometer
OD	optical density

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PknG	protein kinase G
PtpA	protein tyrosine phosphatase A
PWM	pokeweed mitogen
qPCR	real-time polymerase chain reaction
RBC	red blood cell
ROC	receiver operating curve
Se	sensitivity
Sp	specificity
S/P	sample to positive ratio
Th	T helper lymphocytes
U	unit
UBC	University of British Columbia
µg	microgram
µl	microliter
WBC	white blood cell

CHAPTER 1. GENERAL INTRODUCTION

Johne's disease, also known as paratuberculosis, manifests as an infectious enteritis in dairy cattle; other ruminants; and several species, including wildlife, exotic animals, rodents, and birds (Fecteau and Whitlock, 2010; Manning and Collins, 2010b). The characteristic chronic nature of this disease is caused by the intracellular agent, *Mycobacterium avium* subsp. *paratuberculosis* (**MAP**), and its hallmark ability to avert normal immune attempts to clear the infection (Bannantine and Stabel, 2002). Paratuberculosis has plagued the dairy industry since the 19th century, gaining the familiar title of Johne's disease in 1906, following the research efforts of Dr. Heinrich Albert Johne and his colleagues (Manning and Collins, 2010a). Since then, ongoing, widespread research has been pursued to understand the complex nature of this organism and the challenges to control it amid serious concerns of a potential zoonotic link with human Crohn's disease.

1.1 Johne's Disease in Dairy Cows: Impact on the Dairy Industry

1.1.1 Prevalence

Although it has a worldwide distribution (Collins, 2003; Singh et al., 2013), paratuberculosis has become endemic in most of Europe and North America where regions have more than 50% of herds infected (Nielsen and Toft, 2009). A recent study in U.S. herds reported a herd prevalence of 91% (Lombard, 2011). However, due to

imperfect diagnostic tests, prevalence reports may vary, depending on the choice of diagnostic method and target condition, often making comparison between studies difficult (Barkema et al., 2010). For example, herd-level seroprevalence has been reported as 22% of approximately 1,000 U.S. dairy herds (Wells and Wagner, 2000) and 17% of 90 Canadian Maritime herds (VanLeeuwen et al., 2001). Using milk ELISA testing, a recent study in Ontario, Canada, estimated 26% of over 2,000 herds to be MAP-infected (Ontario Johne's Education and Management Assistance Program, 2013). Using environmental fecal testing, 70% of herds in Alberta were classified as MAP-infected (Alberta Johne's Disease Initiative, 2014), whereas 26.5% of 457 participating herds in Atlantic Canada were shown to be MAP-infected, at least once during a three year voluntary program from 2011-2014 (MacDonald et al., 2014).

Within-herd prevalence has generally been reported as low to moderate (Lombard, 2011). The above studies have shown at the cow-level that 2.6% of Canadian Maritime cows (VanLeeuwen et al., 2001), 3.4% of U.S. cows (Wells and Wagner, 2000), and 1.0% of cows in Ontario (Ontario Johne's Education and Management Assistance Program, 2013) to be infected with paratuberculosis, respectively. However, McKenna et al. (2004) reported a cow-level prevalence of 16.1% in eastern Canada using tissue culture.

1.1.2 Economics

Because this disease can be transmitted silently and often goes undiagnosed, it can have long-term effects within a herd. Dairy Farmers of Canada (2014) currently lists Johne's disease as one of the top two animal health priorities of the Canadian dairy

industry. In dairy cattle, Johne's disease is a production limiting disease, leading to direct financial losses for dairy farmers, including veterinary costs (Benedictus et al., 1987), decreased milk production of up to four kilograms less milk per day (Lombard et al., 2005), reproductive losses (Johnson-Ifearulundu et al., 2000), mortality, and culling (Ott et al., 1999). The costs for these direct losses, not including indirect losses from trade implications and potential zoonotic risks, were estimated in the National Animal Health Monitoring System's dairy study (1996) for the United States to be U.S.$200-250 million annually (Wells and Wagner, 2000). Comparatively, recent Atlantic Canadian estimates suggest that a production loss alone from paratuberculosis infection in a 100 cow herd could amount to $5,000 annually (Chi et al., 2002). Unfortunately, depending on the prevalence of the disease within a herd, benefits of implementing management strategies can take up to five to ten years before a noticeable economic effect is felt (Barkema et al., 2010). At the cow-level, control efforts made on the basis of testing can result in benefits that generally outweigh the cost of testing (Dorshorst and Lombard, 2006).

1.1.3 Zoonotic: Crohn's Disease

Public health concerns of a zoonotic link of the causative organism in Johne's disease to Crohn's disease, incurable chronic enteritis in humans, exist. The same pathogen causing Johne's disease in cattle has been identified from patients with Crohn's disease, leading to concerns of decreased milk demand in the market (Hermon-Taylor and Bull, 2002; Gill et al., 2011; Serraino et al., 2014). Although a definitive connection has not been proven between Johne's disease in cattle and Crohn's disease in

humans, the debate still continues on whether MAP may be causally associated with Crohn's disease or is merely a complicating bacteria to the condition (Feller et al., 2007; Chiodini et al., 2012). The concern also involves the possibility of milk as a vehicle of transmission to humans, particularly since it has been shown that, to a certain extent, MAP can survive some pasteurization techniques for milk (Thompson, 1994; Grant et al., 2002; Manning and Collins, 2010b; Van Brandt et al., 2011). Furthermore, several studies were found to report, in some cases, high numbers of the bacteria in dairy products such as milk, cheese, and infant formula (Grant, 2010). It has also been shown that MAP can be transmitted by run-off from MAP contaminated environments into water supplies, with a risk of MAP resistance to water disinfection techniques (Grant, 2010; Manning and Collins, 2010b). The prospect of a confirmed zoonotic link between paratuberculosis and Crohn's disease and the devastating economic implications for the dairy industry continuously drives ongoing paratuberculosis research for improved diagnostic efficiency and control scheme efficacy.

1.2 Johne's Disease in Dairy Cows: The Disease

1.2.1 Overview

Johne's disease is caused by MAP, an obligate intracellular, acid fast bacteria from the *Mycobacterium avium* complex (He and De Buck, 2010), but with specific, detectable genes that differentiate it from other mycobacteria within this complex (Green et al., 1989; Ellingson et al., 1998). Adult cows older than two years are the main source of bacterial shedding, mostly via feces, but also through milk, colostrum, and *in utero*, depending on the stage of infection. Once a susceptible animal has become infected, the

disease progresses slowly. Traditionally, stages one and two of the disease are preclinical periods, while stages three and four have overt clinical signs (Fecteau and Whitlock, 2010). More recently, Johne's disease stages have been tiered into three categories, namely: cows that carry the MAP bacterium without detectable shedding (infected); cows that are shedding a detectable amount of MAP at the time of diagnostic testing (infectious); and cows that exhibit clinical signs of the disease (affected) (Nielsen and Toft, 2008). This new classification system combines the traditional third and fourth stages into the affected group, and combines stage two and stage three cows that have detectable shedding into the infectious group. The following sections explain Johne's disease using both the traditional and newer categorization methods.

1.2.2 Transmission

Immune-suppressed cows and those exposed to a high burden of bacteria can become infected; but, while there is more often resistance to infection with increasing age (Larsen et al., 1975; Chiodini et al., 1984), the specific age of diminished susceptibility has not yet been determined. In a metanalysis study, it was suggested that cows infected as adults may not reach the clinical stage in their lifetime (Windsor and Whittington, 2010).

MAP infection occurs primarily in calves, with a higher susceptibility reported for calves younger than one year and especially younger than six months (Windsor and Whittington, 2010; Mortier et al., 2013), with the highest risk of infection for those less than one month of age (Sweeney, 2011). The earliest infection can begin *in utero*, with a potentially higher risk for the fetus from an infectious or affected dam (Fecteau and

Whitlock, 2010). Postnatally, infection is attained primarily through the fecal-oral route (Clarke, 1997), as well as through MAP-infected or MAP-contaminated colostrum, milk, water, feed, dust, soil, or environment (Sweeney, 1996; Eisenberg et al., 2010). Calves are highly susceptible to MAP exposure via manure contamination on the dam's udder while in the maternity pen environment (Fecteau and Whitlock, 2010; Sweeney, 2011). Colostrum, especially from infectious and clinical dams, also presents a high risk of MAP exposure to calves (Nielsen et al., 2008). Aerosolized MAP poses a further risk to calves (Eisenberg et al., 2011). Studies have shown that the higher the exposure rate and concentration, the less likely the exposed calf's immune system can resist subsequent infection and progression of the disease (Begg and Whittington, 2008; Mortier et al., 2013).

1.2.3 Preclinical Silent Stage

Preclinical stages start at infection and progress through a silent (non-detectable) stage into infectious stages. Because the incubation period between infection and clinical symptoms can range from two to ten years (Whitlock and Buergelt, 1996), some cattle remain within the preclinical phase, while other animals, usually exposed to a higher MAP bacterial load, may take less time to reach the clinical state (Lombard, 2011). MAP-infected cows in this stage usually do not shed MAP bacteria in their feces (silent infection) or have detectable antibodies to MAP (Whitlock and Buergelt, 1996). However, an inflammatory response via macrophages and lymphocytes within the small intestinal, primarily ileal, submucosa and mesenteric lymph nodes creates the formation of granulomas in the intestinal tract as an attempt to control the infection (Sweeney,

2011). Therefore, the presence of infection at this stage can only be confimed via histopathology and culture of the intestinal tract and regional lymph nodes. Although lacking specificity for Johne's disease, Johnin skin tests and interferon-gamma (**IFN-γ**) tests detect these early cell-mediated immune responses and can also be used in this disease stage as an indirect detection method by determining exposure to the disease and subsequent risk of becoming MAP-infectious (Tiwari et al., 2006).

1.2.4 Preclinical Infectious Stage

Progression to this stage, still within the preclinical phase, begins when the cell-mediated attempt to contain the infection fails (Sweeney, 2011). As the cell-mediated immunity wanes, a switch to humoral antibody immunity ensues (Stabel, 2000). Progression of infection increases, resulting in MAP shedding into feces and into other tissues (Whitlock and Buergelt, 1996; Sweeney, 2011). However, this bacterial shedding is intermittent rather than continuous (Nielsen, 2008), as MAP-infected macrophages within intestinal granulomas are shed across the mucosal lining into the intestinal luminal space (Sweeney, 2011). This migration of MAP-infected macrophages can decrease during periods of regression, resulting in waxing and waning phases of bacterial shedding (Sweeney, 2011). Therefore, a negative fecal detection test result at this stage does not necessarily imply that the cow is Johne's disease-free, but rather that shedding was not occurring at that one time or that the bacteria in that particular sample were fewer than the detectable limit. False negative test results are caused by poor diagnostic sensitivity (**Se**) for preclinical cows, although MAP still silently contaminates the environment, increasing risk of exposure to calves (Tiwari et al., 2006). Although

rare, it is also possible that cows in the preclinical stages can have an antibody response detected via an enzyme linked immunosorbent assay (**ELISA**) (Nielsen, 2008; Sweeney, 2011). Higher antibody concentrations typically correspond to increased MAP shedding and consequently disease progression (Koets et al., 2001).

1.2.5 Clinical Infectious Stage

The transition from preclinical to clinical stages can begin as early as two years or up to ten years post-infection, but most commonly between three to five years (Larsen et al., 1975; Whitlock and Buergelt, 1996). This disease transition can be accompanied by both reduced milk production, which sometimes can be noticed up to 300 days before any detectable humoral response by ELISA (Sweeney et al., 2006), and reduced reproductive efficiency, including days open and repeat breeding (Fecteau and Whitlock, 2010). As an infected cow moves into this stage, weight loss and diarrhea commonly ensue (Whitlock and Buergelt, 1996). Similar to shedding, diarrhea begins intermittently and worsens over time. To compensate for fluid lost with the diarrhea, polydipsia may also be observed. Nevertheless, heart rate, respiratory rate, temperature, and appetite remain within normal limits. These clinical signs occur as the focal ileal granulomas become disseminated and spread to other parts of the intestinal tract leading to a thickened intestinal lining, with subsequent malabsorption and protein loss (Stabel, 1998). As the clinical cow's immune system becomes overwhelmed, bacterial shedding increases dramatically not only in feces but also within milk, colostrum, the uterus, muscle tissue, and internal organs. Often stressful situations, including parturition, can be followed by increased or more obvious clinical signs and further bacterial shedding

(Fecteau and Whitlock, 2010). Ultimately, the advanced clinical stage presents with profuse watery diarrhea, hypoproteinemia leading to subcutaneous edema (usually submandibular), lethargy, and severe reduction in milk production. Cows that are not culled prior to stage 4 become debilitated and die from emaciation and dehydration (Sweeney, 2011).

Since milk production loss causes many infected cattle (between 85 to 90%) to be culled during preclinical stages, few infected cattle may reach the clinical stage (Abbas et al., 1983). However, adult cows with clinical symptoms of MAP can shed 10^6 to 10^8 colony forming units (**CFU**)/gram of feces (Jørgensen, 1982; Whittington et al., 2000). MAP shedding into milk has been reported as less than 10 CFU/50 ml of milk for infected cows (Sweeney et al., 1992; Rademaker et al., 2007), versus up to 100 CFU/ml of milk for affected clinical cows (Giese and Ahrens, 2000). However, a calf can become infected with Johne's disease by ingesting merely 50 to 10^3 CFU of MAP/calf (Chiodini, 1996; Gilmour et al., 1965).

1.2.6 Host responses: Cell-mediated and Humoral Immunity

Following exposure and infection with MAP, the pro-inflammatory cell-mediated immune system in susceptible hosts is activated. Macrophage-surface major histocompatibility complex (**MHC**) class II molecules are involved in the presentation of the bacteria to T helper (**Th**) cells. These activated T cells, in particular Th1 cells, such as CD4+, CD8+ and γ/δ cells, and natural killer (**NK**) cells (Stabel, 1996), lead to secretion of IFN-γ. IFN-γ stimulates the production of Immunoglobulin (**Ig**) G2,

activates macrophages, and induces MHC class II expression on the macrophage surface. As the incubation period for the disease progresses and an anti-inflammatory state unfolds, a gradual switch to a Th2 immune response leads to a humoral response and the production of IgG1 antibodies with the onset of clinical signs (Nielsen and Toft, 2006; Stabel, 2010; Sweeney, 2011). Recent research into this transition suggests that rather than the classical Th1/Th2 explanation (which still remains an invalidated hypothesis), the transition may be more likely affected by cytokines such as interleukin (**IL**)-10 from regulatory T cells or IL-17 from proinflammatory Th17 cells (Dudemaine et al., 2014). It has been suggested that the transition to humoral immunity, when the host loses control over the infection, can occur as early as 10 to 17 months old (Lepper et al., 1989), but is more typically seen between 2 to 6 years of age (Nielsen and Ersbøll, 2006). This humoral immune response is delayed following infection because of MAP's survival within macrophages (Hostetter et al., 2003; Kabara and Coussens, 2012).

Because of the intracellular proliferation of MAP, cell-mediated immune response is one of the main pathways to respond to bacterial invasion (Stabel, 1996). It has been suggested that cows that never will be ELISA positive still have a 2 to 4% chance of being fecal culture positive. Therefore, most infected cows will also eventually show a detectable humoral response, although it commonly occurs during infectious stages (Nielsen and Toft, 2006). MAP-infectious cows that are positive for both humoral antibodies and cell-mediated IFN-γ may be less likely to progress to affected stages than those that are only antibody-positive (Jungersen et al., 2012).

It is the hallmark intracellular state of MAP that causes the chronic nature of the disease. In a normal immunological pathway, engulfed microorganisms are enclosed in

vesicles termed phagosomes, transported towards lysosomes, and killed. Processed antigens are then presented to specific Th cells. Since MAP multiples in macrophages, it manipulates the immunological response of the macrophage (Li et al., 2005), allowing the bacteria to not only survive but multiply within the host's macrophages (Bannantine and Stabel, 2002). Intracellular MAP survivability is mediated by the prevention of phagosome-lysosome fusion within macrophages and the decrease of phagosome acidification (Walburger et al., 2004; Bach et al., 2008; Wong et al., 2011). By altering normal macrophage activity, MAP can inhibit normal antigen processing and presentation and alter innate immune activity. For example, MAP can reduce MHC class II levels in these infected macrophages, thereby decreasing the amount of antigen presented to and the activation of Th cells and subsequently the production of IFN-γ (Bach et al., 2011; Sweeney, 2011; Verschoor et al., 2010; Dudemaine et al., 2014). Research is ongoing into identifying and classifying the battery of proteins secreted by MAP in order to cleave lipids and allow basic metabolism and survival for the bacteria (Bach el al., 2011). Two of the identified proteins released upon macrophage-uptake of MAP are protein tyrosine phosphatase A (**PtpA**) and protein kinase G (**PknG**) (Walburger et al., 2004; Bach et al., 2006). In general, protein kinases activate protein substrates by mediating phosphorylation, but the protein phosphatase action of dephosphorylation (specifically of tyrosine amino acids in the case of PtpA of MAP) is a reverse-regulation of kinases to prepare for the next signal transduction (Av-Gay and Everett, 2000; Bach et al., 2006; Bach et al., 2009). The important virulence capability of tyrosine phosphatases, in addition to involvement in many cellular activities (Charbonneau and Tonks, 1992; Walton and Dixon, 1993), highlights their role in the

survival of MAP within macrophages (Bach et al., 2006). PtpA is actively involved in disruption of phagosome-lysosome maturation by reducing the pH in MAP-containing phagosomes through exclusion of the H+ATPase-subunit H, whereas PknG can inhibit phagosome-lysosome fusion and thereby aid the bacteria in avoiding hydrolases from the lysosomes (Walburger et al., 2004; Bach et al., 2008; Wong et al., 2011).

1.3 Johne's Disease in Dairy Cows: Diagnostic Assays

1.3.1 Overview

Due to the chronicity and preclinical nature of the disease, no single assay at a time will be able to detect all infected cows within a herd (Kalis, 2003). Furthermore, even when more sensitive molecular assays are used, fewer than one third of infected preclinical cows in a herd will be identified (Whitlock, 2009). This leads to what has been dubbed the "iceberg phenomenon" for paratuberculosis, in which the few diagnosed affected cows are merely sentinels of the majority of the undetectable MAP infection residing within a herd. In fact, for every stage 4 cow within a herd, there can concurrently be 15 to 25 cows within stage one and another six to eight within stage two (Whitlock, 1992; Fecteau and Whitlock, 2010). The proportion of detectable animals within this pyramid increases dramatically with increasing age, from 33% for two year old cows to 94% for five year old cows (Nielsen et al., 2013). This would indicate that the greater the number of these older cows at the tip of the pyramid, the greater the MAP infection burden within the herd.

There are three broad categories of diagnostic tests available for Johne's disease detection and monitoring: cell-mediated assays, humoral assays, and organism detection

assays (Barkema et al., 2010). The most commonly used methods include culture and polymerase chain reaction (**PCR**) to detect the bacterium, and ELISA to detect immune responses. Individual tests available for Johne's diagnostics perform better as the stage of the disease increases (Tiwari et al., 2006).

1.3.2 Culture

For MAP, culture can be used not just for feces, but also milk, colostrum, and tissues (intestine, muscle, lymphoid). Despite the intermittent shedding of MAP in preclinical phases, fecal culture is considered in much of the literature as the primary reference standard, as tissue culture is invasive and therefore reserved mainly for post-mortem detection (Bölske and Herthnek, 2010). Unfortunately, MAP is extremely slow growing, and culture incubation times for bovine samples can take up to seven weeks on broth media and up to 16 weeks on solid media. This slow growth of MAP necessitates the use of decontamination techniques, including antimicrobials, to eliminate faster-growing, competitive bacteria and fungi (Whittington, 2010), for which there are various media and techniques available. Commonly used solid media includes Herrold's egg yolk media (**HEYM**) with the addition of mycobactin J, an iron-chelating agent, since MAP is the only mycobacterium that can use mycobactin J as a siderophore. A benefit to solid media is the direct detection and enumeration of MAP colonies which gives an indication of bacterial load. Liquid media, on the other hand, is quicker, and qualitative or quasi-quantitative. Liquid systems include the Bactec 460 radiometric detection, Bactec MGIT 960 fluorescence detection, TREK® ESP pressure detection, and MB/BacT reflectance detection (Whittington, 2010). For milk and colostrum samples,

where bacterial numbers tend to be much lower than in feces, sedimentation, centrifugation, and filtration are all extra steps that can be added to the culture protocols to aid in bacterial identification (Gao et al., 2005; Gao et al., 2009; Bradner et al., 2013a). In addition, to decrease costs, culture of environmental fecal samples, and/or pooled fecal samples is a common practice for herd testing and screening, where the number of individual cows/pool depends on the total number of cows within a herd. Positive-pool culture results can then be followed up with the culture of individual samples to identify positive cows within that pool (Whittington, 2010; Lavers et al, 2013). However, the culture techniques require further confirmation of MAP growth with positive acid fast staining and with molecular identification methods using MAP specific genes.

Fecal culture specificity (**Sp**) is nearly 100% (Nielsen and Toft, 2008), while Se of 26% for pre-clinical cows and 82% for infectious cows have been reported (McKenna et al., 2005; Sockett et al., 1992; Whitlock et al., 2000). Tiwari et al. (2006) reported Se of fecal culture from19% in low MAP-shedding cows to 53% in high MAP-shedding cows. Herd level screening and decreased cost can be achieved with the pooled fecal culture method (pools of five cows) (Kalis et al., 2004) and environmental fecal cultures.

Unfortunately, Se of culture methods for individual milk and colostrum samples or even bulk tank milk samples would invariably be lower than for feces, as there is a significantly smaller proportion of MAP-fecal shedding cows that are suspected to shed the bacteria through their mammary glands (Jayarao et al., 2004; Streeter et al., 1995; Sweeney et al., 1992). In addition, milk results need to be cautiously interpreted as they risk being biased by potential fecal contamination (Barkema et al., 2010).

1.3.3 Polymerase Chain Reaction

As an alternative to direct growth and identification of the bacterium, molecular techniques can be used to detect the bacterium genetically. Polymerase chain reaction methods have been developed for feces, milk, colostrum, and tissue samples directly or as confirmation to culture techniques. In addition, the quasi-quantitative cycle threshold (**Ct**) values obtained can be used to estimate the amount of MAP present in the sample, with lower Ct values corresponding to higher MAP concentration as fewer amplification cycles are required for the fluorescence to reach the required threshold (Bölske and Herthnek, 2010).

There are 3 main advantages to PCR, particularly real-time PCR (**qPCR**). First, it is a very rapid technique compared to culture, taking less than 24 hours if needed to obtain results. Second, no decontamination techniques are required, which avoids the subsequent reduction in viable MAP numbers within the sample that is seen with culture methods. And third, PCR can detect not only viable bacteria, but also dead bacteria, which is helpful when fresh samples cannot be processed immediately, as some bacteria may be killed with particular storage methods (Bölske and Herthnek, 2010). The insertion element *IS900* is the usual sequence targeted; however, sequences such as *hspX*, *F57*, *ISMAV2*, and *ISMAP02* are unique to MAP (Ellingson et al., 1998; Bölske and Herthnek, 2010; Pithua et al., 2011; Hanifian et al., 2013), allowing Sp to be comparable to culture techniques (Leite et al., 2013). Sensitivity of the molecular method will be dependent on technique and kit used, gene identified, reference standard, bacterial load within the sample, and loss of bacteria during processing or storage

(Bölske and Herthnek, 2010; Leite et al., 2013; Plain et al., 2014). Another important factor that can affect the Se and Sp of the assay is the number of copies of the selected gene sequence, with Se increasing with greater copy numbers. However, concern over cross-reaction with other mycobacteria harbouring *IS900*-like elements have led to the selection of other MAP-specific sequences, despite their having fewer copy numbers (Bölske and Herthnek, 2010). Furthermore, the selected cut-point for a positive result can lead to an underestimated Se the lower the cut-point threshold. Therefore, comparisons of qPCR results for paratuberculosis research across the literature need to be cautiously interpreted in light of these factors. A disadvantage to the efficiency of PCR for paratuberculosis is the challenge faced in extracting DNA from MAP, which can be enhanced by selecting the most accurate methodology for the purpose of testing (Leite et al., 2013). Two specific challenges faced during DNA extraction from MAP include inhibitors present in the sample that can hinder DNA amplification, such as phytic acid and polysaccharides within fecal samples (Kreader, 1996; Monteiro et al., 1997; Thornton and Passen, 2004; Leite et al., 2013), and also the thick, waxy cell wall of MAP, which requires more intense lysis techniques, such as bead-beating, to extract the DNA (Lanigan et al., 2004; Leite et al., 2013).

1.3.4 Enzyme Linked Immunosorbent Assays

The second group of diagnostic tests involves detecting an immune response to the bacteria, with ELISA most commonly used. ELISA kits can be used to detect a quantitative optical density (**OD**) reading that correlates to the amount of MAP-specific antibody in either serum or milk samples (Nielsen, 2010). This assay is widely used,

being cost-effective and taking only a few hours to process. The Se of ELISA is generally poor but varies greatly depending on reference standard, target condition, herd MAP prevalence, and ELISA kit and methodology (Nielsen et al., 2002; Collins et al., 2005; Lombard et al., 2006; Nielsen and Toft, 2006). For example, McKenna et al. (2005) reported Se of absorbed serum ELISA as low as 6.9% to 8.8%, but Se of unabsorbed ELISA at 16.9%, when tissue culture is used as the reference standard. Meanwhile, Sp for ELISA has been shown to be less than 100% depending on the kit and method (McKenna et al., 2005; Nielsen and Toft, 2008). Therefore, ELISA results (milk or serum) need to be evaluated in light of the purpose for testing (such as detection or screening) (Nielsen and Toft, 2006), and should be followed up with direct detection methods for absolute confirmation (Collins, 2011).

Furthermore, there can be much variation between ELISA kits and protocols, with results ranging from strong positive in one type of commercial ELISA to negative in another on the same sample. One reason for this lies in the unique antibody responses within each cow (Collins et al., 2005). The detection of antibodies to IgG1 occurs as the pro-inflammatory stage (IgG2) switches over to the humoral anti-inflammatory stage (IgG1). Sometimes both IgG1 and IgG2 can occur simultaneously during the immune transition (Th1 to Th2 shift) (Nielsen, 2010). In addition, the type of IgG antibody detected depends on the sample. For example, IgG1, which is primarily detected in milk and colostrum, only characterizes approximately half of the IgG present in serum. Therefore, differences among Se of ELISA kits and protocols may also be dependent upon the target condition and sample (Harp et al., 1988).

In a study by Sorge et al. (2012) of dairy herds in Ontario and Western Canada, the two most important risk factors for a positive milk ELISA test outcome included MAP herd status and Johne's disease history. The effect of these risk factors may also potentially affect the risk of a positive outcome using other diagnostic tests for paratuberculosis. Other risk factors listed by Sorge et al. (2012) included the introduction of subclinical or unknown status cows into test-negative farms, exposing young calves to the bacteria, increased use of pooled colostrum, calf management, nutrition, and disease states.

1.3.5 Interferon Gamma Assays

Detection of IFN-γ, although not necessarily always indicating MAP infection, can be used in the context of a "recall" immune response. This recall IFN-γ response identifies cows previously exposed to MAP and at potential risk of later disease development and risk of future MAP transmission occurring in the herd (Jungersen et al., 2002; Huda et al., 2003; Huda et al., 2004; Jungersen et al., 2012). Specifically, this assay identifies the host's T cell recognition of antigens since the cows may not have been infected long enough to produce antibodies to MAP (Stabel and Whitlock, 2001; Nielsen and Toft, 2006; Zervens et al., 2013). Although earlier than other tests, IFN-γ detection of MAP may still lag by several months following infection (Chiodini, 1996). Although Begg et al. (2011) reported that in MAP-infected sheep different immune profiles may exist in early infection, for MAP-infected cows, the IFN-γ assay may have improved performance in MAP-infected cows less than three years of age, in comparison to antibody ELISA (Huda et al., 2004; Jungersen et al., 2012).

Unfortunately, this assay has never gained widespread use in Johne's diagnostics due to two major disadvantages to the test that require further research to overcome. The primary disadvantage is the need to process fresh whole blood samples within 24 hours of collection in order to maintain white blood cell viability, with improved results for samples processed within eight hours of collection (Jungersen et al., 2002; Plain et al., 2012). Furthermore, by sensitizing lymphocytes through the action of specific stimulation agents, the recall-response leads to the production of IFN-γ, which can be detected through cell-mediated assays. A common sensitization agent for this assay is Johnin, a protein-purified derivative antigen. However, the Se and Sp of the assay to identify the MAP-infected cow's T cell recognition of antigens is often poor and quite variable, especially when using Johnin (Jungersen et al., 2012). With the potentiating effects of the pro-inflammatory cytokine IL-12 or of anti-IL-10 (an anti-inflammatory cytokine) antibodies added to the culture with a stimulation agent, a greater possible Se of the assay could be achieved (Jungersen et al., 2005; Mikkelsen et al., 2009; Mikkelsen et al., 2012). Detection of IFN-γ in exposed animals does not necessarily mean MAP infection will ensue, as the cell-mediated immunity may control the infection or the IFN-γ may be only detecting environmental mycobacteria, lowering test Sp, if the antigen selected is not highly MAP-specific (Jungersen et al., 2002; Huda et al., 2004; Jungersen et al., 2012). As cell-mediated responses are strong during early infection, IFN-γ testing could be used as a support mechanism to identify gaps in paratuberculosis management protocols that result in MAP exposure, especially for subclinical cows and calves (Collins, 1996; Kalis et al., 2003).

1.4 Johne's Disease in Dairy Cows: Management and Control

Even the best diagnostic test for paratuberculosis is likely to misdiagnose as false negative a large number of infected cows within a herd (Whitlock, 2009), which is one of the reasons eradication of paratuberculosis from a farm is so difficult (Collins et al., 2010; Whitlock, 2010). For those cows correctly identified as MAP-positive, there is no currently effective treatment, cure, or vaccine available (Whitlock, 2010). Although some therapeutic agents have been shown to decrease clinical signs, they are not curative and not economically feasible for large-scale use, as they need to be administered for the life of the cow to be of benefit (Fecteau and Whitlock, 2011). Vaccines can be used concurrently with control measures, and in some cases, as for small ruminants, have been shown to be a cost-effective means for controlling clinical signs of Johne's disease, especially decreased milk production (de Lisle, 2010). However, for bovine paratuberculosis, some problems still decrease the use of vaccines as part of control measures. In Canada, there is currently no approved paratuberculosis vaccine, primarily due to the interference with bovine tuberculosis skin testing (Patton, 2011), as well as potential false positive results in paratuberculosis immune tests (de Lisle, 2010; Tewari et al., 2014). Approximately 5.0% of dairy herds in the United States incorporated vaccination in their Johne's control programs in the National Animal Health Monitoring System study from 2007 (NAHMS, 2007). Furthermore, although vaccines can significantly delay and decrease clinical signs and fecal shedding (Tewari et al., 2014) and thereby synergistically benefit control programs, they do not prevent infection in herds and can cause the formation of large granuloma reactions at the inoculation sites (de Lisle, 2010).

Consequently, once MAP has been detected within a herd, management and biosecurity are currently the primary means of control. Likewise, screening and biosecurity are a priority for test-negative herds to maintain this status and prevent MAP from entering the herd (Whitlock, 2010; Garry, 2011). In this manner, the most cost-effective approach to reduce herd prevalence and manage Johne's infection within a herd is to identify where transmission is most likely occurring and interrupt this transmission of MAP through specific control protocols (Groenendaal et al., 2002; Kudahl et al., 2007; Nielsen et al., 2008).

There are two main goals for management programs, namely: decreased transmission to calves and heifers, and prevention of disease introduction into herds (Whitlock, 2010). With regard to biosecurity, a closed herd is the best option. But if replacement heifers are purchased, then it is necessary for the replacements to come from herds that are serially test-negative. For a herd that is already infected with MAP, there can be several critical control points specific to that herd, including maternity pen management, as calves are at the highest risk for infection. Known MAP-positive or high risk cows should be segregated from the maternity area to avoid MAP-contaminated feces in that environment. The maternity area should be kept clean and dry, and calves should be removed from dams immediately after birth (Whitlock, 2010). Calves should not be fed pooled colostrum, nor colostrum and waste milk from infected cows (Chiodini and Hermon-Taylor, 1993). In addition, young stock and heifers should also not be fed MAP-contaminated feed and water. Because fecal shedding and subsequent ingestion is a major transmission mode, manure management, particularly in the maternity pens and amongst dry cows, becomes a priority. Best management

practices, as suggested by the National Voluntary Bovine Johne's Disease Control Program, should include education for the producer, preferably as part of a national or provincial voluntary control program, and the development of a risk assessment and management plan that is unique to each herd, followed by specific decisions for testing programs to detect and monitor MAP-positive cows. A cost-benefit analysis is often at the core of specific control objectives (Collins et al., 2011; Garry, 2011; Whitlock, 2010).

1.5 Research Limitations

Current ante-mortem diagnostics are inadequate to meet the industry's needs for efficient identification of preclinical stages, herd biosecurity, and identification of MAP transmission and transmission control efficacy (Nielsen and Toft, 2008). Difficulty due to inconsistent MAP detection can impair the biosecurity-based management programs implemented in a herd (Whitlock and Buergelt, 1996; Harris and Barletta, 2001; Bannantine et al., 2004). Although research into numerous facets of paratuberculosis is underway, knowledge gaps and limitations still exist, particularly in the diagnostic arena. One of the greatest limitations paratuberculosis research and diagnostic study faces is the lack of highly sensitive and reliable testing methods due to the nature of the disease (Nielsen and Toft, 2008; Whitlock, 2009). Being able to identify patterns in MAP shedding and factors affecting progression of the disease, and consequently increased MAP shedding, are important to implementing cost- and time-efficient and effective management and control programs at the cow and herd levels, while continued research into therapies and control measures are underway. The urgency of the need to

reach these goals is fuelled by the rising concern of a zoonotic link between bovine paratuberculosis and human Crohn's disease, and the potential subsequent devastating effect on the dairy industry (Herman-Taylor and Bull, 2002; Gill et al., 2011; Chiodini et al., 2012; Serraino et al., 2014).

Understanding patterns of MAP shedding detected by immune-based and bacterial detection assays can lead to more time- and cost-efficient diagnostic strategies. Little knowledge of any MAP shedding patterns over time, particularly at the cow-level, has been documented or developed. There is also a lack of comparable scientific research on MAP shedding and immune patterns during lactation and over seasons in dairy cows. This knowledge gap can impede advances in the appropriate use of diagnostic tests in current MAP control programs within the dairy industry. Such knowledge can assist in the creation of herd- and time-specific control programs.

A second limitation lies in the current diagnostics' inability to efficiently and reliably identify early MAP infection in order to decrease the number of cows silently transmitting MAP within a herd (Kalis et al., 2003). The imperfect and inconsistent detection abilities of the assays deter producers from readily participating in control programs. The more accurately and efficiently the test results can be obtained, the more producer involvement in early and continued control measures will likely improve. One of MAP's hallmark survival mechanisms is to evade the host's natural immune pathways, so the earlier the infection can be diagnosed, the sooner specific, efficient control mechanisms can be instigated (Stabel et al., 2007). Although research is ongoing in this area, the development of early and effective diagnostic assays is still required. Investigation for early and universally available MAP-specific proteins and antigens to

serve as reliable biomarkers of the disease (Bannantine et al., 2004) is an ongoing and necessary effort to characterize them as useful diagnostic tools and reagents.

1.6 Thesis Objectives/Focus of Research

The focus of this research was to improve MAP control and management programs through increased understanding of the diagnostic detection ability of common pathogen and antibody assays over seasons and lactation stages, as well as through the novel use of MAP-specific proteins and a cell-transport media for early diagnostic potential.

Due to the imperfect reference standard of fecal culture for paratuberculosis, a clear description of the target condition and the purpose of testing are required to adequately interpret test results (Nielsen and Toft, 2006). For studies evaluating diagnostic tests, a case definition can practically define the target condition (Gardner et al., 2011). For Chapters 2 to 5, the target condition was a MAP-infectious cow. A case was defined as a cow that had detectable fecal shedding at least once, as determined in a companion study (Lavers et al., 2013), in a one-year period prior to the start of the study. Although defining the transition from infected to infectious stages can be ambiguous at times, for this research, MAP-infectious was chosen, following the guidelines suggested by Nielsen and Toft (2008) and Gardner et al. (2011). They define a MAP-infectious cow as one that is actively shedding MAP in a detectable amount, as identified by the test under evaluation, and in an amount that can transmit disease to a non-infected cow. Although MAP-infectious cows are a sub-group of MAP-infected cows, since we were concerned with the detection ability of various assays in known MAP-positive cows, a MAP-infectious definition fit within the bounds of our goals more constructively than

MAP-infected. The fecal pathogen detection method employed for case identification in our study (Chapters 2 to 5) was the method used for fecal culture identification, confirmed with acid fast stain and qPCR, as used in the study by Lavers et al. (2013).

1.6.1 Patterns in Detecting MAP Shedding: Feces

Efficient diagnostic strategies for identification of infected cows and monitoring of infectious and affected cows can enhance best management practices to reduce transmission of MAP (Nielsen and Toft, 2006), particularly in the maternity pens and amongst dry cows. However, difficulty with MAP detection due to imperfect assays can impair these biosecurity-based management programs. Understanding patterns of MAP shedding detected by the most commonly used diagnostic tests can lead to their use in a more time- and cost-efficient manner. Seasonal and lactation stage shedding patterns have been assessed previously, but infrequently and with conflicting results (Crossley et al., 2005; Strickland et al., 2005; Norton et al., 2010). This knowledge, however, is still important for the efficient use of fecal diagnostic tests for paratuberculosis detection by controlling for variations in fecal shedding patterns related to time factors. Therefore, the main objective of Chapter 2 was to determine the Se of three commonly used fecal diagnostic methods for paratuberculosis (solid culture, broth culture, and qPCR) and to assess the impact of season and lactation stage on MAP detection.

1.6.2 Patterns in Detecting MAP Shedding: Milk and Colostrum

As with other modes of transmission, milk and colostrum that are infected with MAP pose a threat to calves, which are at the highest risk for infection with Johne's

disease (Lombard, 2011; Sweeney, 2011). A recent meta-analysis of MAP shedding through milk found an overall apparent MAP prevalence of 20% in individual milk samples, as detected with culture and PCR (Okura et al., 2012). Furthermore, a zoonotic concern for milk and its derivatives as a vehicle of MAP transmission to humans (Grant et al., 2002; Manning and Collins, 2010b; Van Brandt et al., 2011) necessitates diagnostic assays for detecting MAP in milk and colostrum as effectively and accurately as possible.

Seasonal differences in MAP recovery from pasteurized milk samples have been reported (Ellingson et al., 2005), but limited information is available regarding the effects of stage of lactation and season on MAP detection in raw milk and colostrum samples (Millar et al., 1996; Grant et al., 2002; Bradner et al., 2013b; Cazer et al., 2013; Stabel et al., 2014). Therefore, understanding patterns of MAP shedding not just in feces but also in milk and colostrum can reveal more time-efficient and cost-efficient detection strategies that can benefit the dairy industry as a whole. The first objective of Chapter 3 was to assess MAP pathogen detection in milk and colostrum with three assays (solid culture, broth culture, and qPCR) and to compare their detection ability versus feces. The second objective was to identify any effects of season or lactation stage on MAP detection within milk and colostrum samples.

1.6.3 Patterns in Detecting MAP Antibodies: Milk ELISA

The presence of antibodies can be predictive of higher risk for MAP fecal shedding (Nielsen, 2008; Lavers et al., 2013). Antibody ELISA is a much more time- and cost-efficient assay than direct pathogen detection methods. However, Se of ELISA

is generally poor (29 to 61%), with Sp between 83 to 100% (Nielsen and Toft, 2008). Improved Se of ELISA should occur with increasing age or parity, as this would typically coincide with increased MAP shedding and clinical signs (Toft et al., 2005; Nielsen et al., 2013). Nielsen et al. (2002) found an increase in Se for milk ELISA at the beginning of lactation, but variation in Se related to lactation stage has to be carefully evaluated in light of Johne's disease stage, the presence of nonspecific colostral antibodies, and milk dilution effects (Nielsen and Toft, 2012). A recent study by Cazer et al. (2013) assessed seasonal effects on bulk tank milk ELISA results and detected increased MAP antibodies in bulk tank milk during summer and a decrease during winter. This effect may be dependent upon seasonal calving or a humoral immunity peak in response to increased exposure to MAP during specific seasons (Collins et al., 2005). The objectives of Chapter 4 were to compare Se of milk ELISA with fecal diagnostic assays (solid and broth culture and qPCR) and to assess how detection of antibody concentrations in milk varies with changes in fecal shedding of MAP, host age or parity, lactation stage, and season.

1.6.4 Novel Early Detection: MAP Specific Proteins

The virulence activities of MAP stem from its ability to thwart immunological attempts to clear the host of the infection and to coordinate the alterations of these complex immune pathways to gain intracellular survival (Bannantine and Stabel, 2002). By secreting virulence proteins within macrophages, MAP is able to survive and replicate. Although the knowledge of which proteins the bacterium secretes is still incomplete, a few proteins have been identified that are secreted and used by MAP for

basic metabolism and survival (Bach et al., 2011). For example, PtpA of MAP, also known as low molecular weight MAP1985 (Li et al., 2005), is secreted early and continually from MAP upon uptake by phagosomes (Bach et al., 2006) and is actively involved in dephosphorylation of a vacuolar sorting protein that is required within macrophages for maturation of the phagosome-lysosome complex (Bach et al., 2008). Similarly, PknG, a eukaryotic-like serine/threonine kinase, is secreted within macrophages following uptake and acts in blocking phagosome-lysosome fusion, thereby preventing the destruction of MAP (Walburger et al., 2004).

Previously, Bach et al. (2011) discovered that an ELISA utilizing PtpA antigen coating was more sensitive in serum of subclinical cows than a commercial antibody ELISA. Therefore, the first objective of Chapter 5 was to determine if this novel ELISA with PtpA or PknG antigen coatings could detect antibodies against paratuberculosis in milk and colostrum samples from the case group of MAP-infectious cows, and ultimately be used as an early cow-side detection method for MAP infection in milk and colostrum samples. This chapter represents the first attempt to assess this novel ELISA protocol on bovine milk and colostrum samples.

Additionally, the cell-mediated IFN-γ assay that measures IFN-γ in serum utilizes antigens like the protein purified derivative Johnin to identify the host's T cell recognition of antigens when cows have not been infected long enough to produce antibodies to MAP (Stabel and Whitlock, 2001; Nielsen and Toft, 2006; Zervens et al., 2013). Because the proteins PtpA and PknG are secreted early, post-infection and within infected macrophages, our second objective for Chapter 5 focused on using these MAP-specific proteins as an alternative to Johnin in IFN-γ assays, as Johnin has historically

shown quite variable activity. Furthermore, the use of specific antigens could increase the Se of the assay, leading to fewer false negative results (McDonald et al., 1999; Bannantine et al., 2004).

1.6.5 Novel Early Detection: Cell Viability

Because cell-mediated blood tests detect subclinically infected animals more readily when the Th1 immune response prevails, they can be sensitive tests for early Johne's diagnostics and control efforts (Stabel, 1996). However, the IFN-γ assay requires processing of bovine blood samples within 24 hours of collection in order to maintain white blood cell viability (Plain et al., 2012). This has caused the test to be less utilized for Johne's diagnostics, even though it is a test for detection of MAP exposure and the presence of IFN-γ in early infection.

Keeping the white blood cells (**WBC**) viable is difficult as on-farm collection and transport to a diagnostic laboratory can often take longer than one day. In a study of *Mycobacterium bovis* stimulated blood, the number of WBCs isolated decreased sharply from fresh to two day old blood at greater or less than room temperature (Senogles et al., 1978). Robbe-Austerman et al. (2006) recommended that for more accurate results, whole blood kept at room temperature should be processed within less than 12 hours of collection. In light of this, the objective for Chapter 6 was to evaluate if using a novel blood cell preservation media (SCSR™, NonInvasive Technologies), previously only used in human medicine, would be a practical method of extending the lifespan of WBCs *in vitro* and, when applied to paratuberculosis diagnostic measures, would allow

for extended whole blood sample transit-time. The benefits of this method could extend beyond paratuberculosis to a variety of other veterinary diagnostic applications.

1.6.6 Summary of Objectives

Overall, the objectives of this research were to determine the cow-level detection ability of four commonly used paratuberculosis diagnostic assays (solid culture, broth culture, qPCR, and ELISA) in cows previously determined to be MAP-infectious. This detection ability was evaluated monthly, over one year, to determine if there was any effect of time (season or lactation stage) on detectable MAP shedding. As early detection of MAP infection is vital to pursuing effective control of Johne's disease within a herd, this research also assessed the use of two early secreted proteins from MAP (PtpA and PknG) as potential biomarkers of (1) MAP infected milk and colostrum, through a novel ELISA method, and (2) exposed or early-infected animals, through a novel IFN-γ method. Finally, to enhance the practicality of the early-use IFN-γ assay for paratuberculosis, a novel blood cell preservation method was evaluated as a means to preserve WBCs *in vitro* for up to eight days, to allow for extended travel times of whole blood samples from collection on-farm to processing in laboratories.

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CHAPTER 2

THE ASSOCIATION OF DETECTION METHOD, SEASON, AND LACTATION STAGE ON IDENTIFICATION OF FECAL SHEDDING IN *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* INFECTIOUS DAIRY COWS

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2.1 Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease. Although fecal cultures are considered the standard diagnostic test, the long incubation times, costs, and intermittent shedding of MAP hinder efficient screening programs. The primary objectives of this study are to determine the detection ability of solid culture, broth culture, and real-time PCR (qPCR) for MAP in fecal samples and to assess how shedding patterns of MAP in feces may vary with lactation stage and season. This knowledge can improve the use of these diagnostic assays in Johne's management programs. For this study, 51 MAP-infectious cows from seven Atlantic Canadian dairy farms had fecal samples collected monthly over a 12 month period. Samples were analyzed for MAP bacterial load via solid culture, broth culture, and qPCR. For all fecal samples, 46% (95% CI: 40 to 51%) were positive with solid culture, 55% (95% CI: 50 to 60%) with broth culture, and 78% (95% CI: 73 to 82%) with qPCR. Sensitivity of qPCR was numerically higher in the dry and postpartum periods, and qPCR detection in summer and fall was 85% of qPCR detection in winter and spring. Furthermore, culture-determined moderate or lighter shedding categories generally corresponded to qPCR cycle threshold (Ct**)** values <35, but heavy shedding categories corresponded with Ct <29. Direct fecal qPCR is a MAP detection method that is quick, less costly than culture techniques, and avoids the use of decontamination steps necessary for culture that can decrease bacterial numbers in a sample to below the detection limit. This study indicates that, for known MAP-positive cows, there was a

high sensitivity of MAP detection with fecal qPCR, thereby supporting the use of direct fecal qPCR as part of a Johne's herd control program.

2.2 Introduction

In dairy cattle, Johne's disease, or paratuberculosis, is an important production limiting disease leading to financial losses for dairy farmers. This economic impact is related to decreased milk production (Lombard et al., 2005), reproductive losses (Johnson-Ifearulundu et al., 2000), mortality, culling (Ott et al., 1999), and increased veterinary costs (Benedictus et al., 1987). Caused by the organism *Mycobacterium avium* subsp. *paratuberculosis* (**MAP**), the disease manifests as chronic enteritis, with an incubation period extending from two to ten years, depending on the infective dose (Whittington and Sergeant, 2001). During the preclinical stage, from initial infection typically as calves, to the time of manifested clinical signs most often as adults, bacteria can be intermittently shed from infected cows and silently spread throughout a farm (Fecteau and Whitlock, 2010). Adult cows with clinical symptoms of MAP can shed 10^6 to 10^8 colony forming unit (**CFU**) per gram of feces (Jørgensen, 1982; Whittington et al., 2000). However, a calf can become infected with Johne's disease by ingesting only 50 to 10^3 CFU per calf (Chiodini, 1996; Gilmour et al., 1965), making the volume of MAP-contaminated feces required to infect a calf extremely low.

Johne's disease status has commonly been tiered into three categories, namely cows that carry the MAP bacterium without detectable shedding (infected), cows that are

shedding at time of diagnostic testing (infectious), and cows that exhibit clinical signs of the disease (affected) (Nielsen and Toft, 2008). Efficient diagnostic strategies for identification of infected cows and monitoring of infectious and affected cows can enhance best management practices to reduce transmission of MAP (Nielsen and Toft, 2006). Since asymptomatic MAP-infected cows can intermittently shed bacteria in their feces, a negative fecal detection test result does not imply that the cow is paratuberculosis free, but rather that the cow was not shedding the organism at the time of testing or that the concentration of bacteria in the sample was below the diagnostic detection limit. Stressful events, including parturition, can increase MAP shedding and precipitate the onset of clinical signs of Johne's disease (Sweeney, 2011).

Despite the intermittent shedding of MAP in preclinical phases of disease, fecal culture is often considered the primary reference standard (Bölske and Herthnek, 2010). Unfortunately, MAP culture incubation times can take up to seven weeks on broth media and 16 weeks on solid media. Furthermore, the slow growth of MAP necessitates the use of decontamination techniques, including antimicrobials, to decrease faster-growing, competitive bacteria and other organisms. Whereas growth in liquid media is quicker than on solid media, a disadvantage of this technique is that the results are only indirectly quantitative (Whittington, 2010). The TREK broth system (Thermo Scientific, Oakwood Village, Ohio) utilizes a pressure detection system to signal positivity, and the days to positive can be used as an estimate of MAP concentration in the fecal sample (Whittington, 2010). In comparison, solid media such as Herrold's egg yolk media (**HEYM**) with mycobactin J, although requiring longer growth times and being more labor intensive, does allow for direct quantitation by a CFU count (Whittington, 2010).

Acid fast stain can be used to confirm that culture isolates have typical morphology. However, PCR of the isolated bacteria provides stronger evidence to confirm MAP over other mycobacteria.

The PCR-based molecular techniques can detect MAP genetically, and the cycle threshold (**Ct**) values obtained can be used to estimate the quantity of MAP DNA present in the sample, with lower Ct values corresponding to higher MAP DNA concentration (Bölske and Herthnek, 2010). More recently, attempts have been made to categorize Ct values in reference to CFU counts obtained via solid culture techniques (Leite et al., 2013). Methods for direct PCR and culture isolate confirmation with PCR have been developed for feces, milk, colostrum, and tissue samples. For real-time PCR (**qPCR**) specifically, there are three main advantages for MAP detection. First, qPCR is very rapid compared to culture. Additionally, the method does not require decontamination steps, as required for culture methods, thereby avoiding any reduction in viable-MAP concentration caused by that process. Finally, qPCR can detect dead as well as viable bacteria, which is helpful when fresh samples cannot be processed immediately as some bacteria may be killed during freezing (Bölske and Herthnek, 2010). Although the insertion element *IS900* has classically been the most common sequence targeted, *hspX* gene is another sequence, a single copy gene, that is unique to MAP (Ellingson et al., 1998; Bölske and Herthnek, 2010) and is used in the Tetracore Real-time PCR kit for Johne's detection (Tetracore, Rockville, Maryland). By using a sequence unique for MAP, specificity (**Sp**) is comparable to culture techniques and can reach 100% (Leite et al., 2013). However, qPCR sensitivity (**Se**) for MAP is dependent upon additional factors including technique, bacterial concentration within the sample,

bacterial loss during processing or storage, and reference standard accuracy (Bölske and Herthnek, 2010).

Because there is no current treatment or cure available for Johne's disease (Whitlock, 2010), a management-based control program is important if a producer wishes to decrease the introduction and spread of MAP within a herd. There are two main goals for management programs, namely decreased transmission to calves and heifers within infected herds and prevention of disease introduction into herds (Whitlock, 2010). Vaccines can be used concurrently with control measures; and in some cases, particularly for small ruminants, have been shown to be a cost-effective means for controlling clinical signs of Johne's disease, especially decreased milk production (de Lisle, 2010). However, in North America, use of the vaccine in dairy control programs is strictly controlled, primarily due to the interference with bovine tuberculosis skin testing (Patton, 2011), as well as potential false positive results in paratuberculosis immune tests (de Lisle, 2010; Tewari et al., 2014). Vaccines can benefit control programs by delaying and decreasing clinical signs and fecal shedding (Tewari et al., 2014). However, they do not prevent infection in herds and can cause the formation of large granuloma reactions at the inoculation sites (de Lisle, 2010). In the fourth National Animal Health Monitoring System study of the U.S. dairy industry (NAHMS, 2007), 5.0% of the dairy herds incorporated vaccination in their Johne's control programs.

Consequently, once MAP has been detected within a dairy herd, management and biosecurity are currently the primary means of control. Because fecal shedding and subsequent ingestion is a major transmission mode, manure management, particularly in

the maternity pens and among dry cows, becomes a priority. Pithua et al. (2013) observed that the use of individual calving pens in Midwestern US dairy herds reduced the subsequent risk of MAP infection for these calves as compared to those born in group calving pens, which underwent variable manure management protocols. Although this effect decreased over time, they recommended the use of individual calving pens, in which strict manure hygiene can be implemented.

Unfortunately, difficulty in identifying MAP-positive cows can impair biosecurity based management programs. As a result, the use of more than one diagnostic method has often been suggested, particularly combining functional immune-based assays and methods assessing MAP excretion into feces, as they are based on different target conditions (Pinedo et al., 2008). Understanding patterns of MAP shedding detected by the most commonly used diagnostic tests can lead to their use in a more time- and cost-efficient manner. Seasonal and lactation stage shedding patterns have been assessed previously but with conflicting results. One study did not find an association between ambient temperature (season) and detection of MAP by fecal culture (Strickland et al., 2005). However, in another study, increased MAP growth was detected in fecal samples collected during winter (November through March) (Crossley et al., 2005). A third study from New Zealand by Norton et al. (2010), found a higher fecal culture detection rate in spring (October). This corresponded with the calving period in that industry, so the effect of season could not be separated from a potential effect of stage of lactation. There is a knowledge gap regarding within-season and within-lactation fecal MAP shedding patterns for culture and molecular methods, especially broth culture and qPCR. However, this knowledge could enhance the efficient

use of fecal diagnostic tests for Johne's disease detection by controlling for variations in fecal shedding patterns related to season or lactation stage.

Therefore, the primary objectives of the study were to determine the Se of three fecal diagnostic methods (solid culture, broth culture, and qPCR) and to assess the impact of season and lactation stage on MAP detection. Secondary objectives included parallel Se usage, agreement evaluation among the diagnostic methods, and the association between Ct values for qPCR and shedding patterns determined by culture techniques.

2.3 Materials and Methods

2.3.1 Farm and Cow Selection

For this study, seven known MAP-positive dairy farms were purposively selected from a companion project assessing MAP herd prevalence in dairy farms in New Brunswick, Nova Scotia, and Prince Edward Island (Lavers et al., 2013). In that study, MAP culture-positive cows were identified following twice yearly herd monitoring with pooled fecal culture. Each pool was comprised of five cows, which were individually cultured if the pool was positive. The recruited farms for our study were selected based on the number of these MAP culture-positive cows as well as proximity to our laboratory to facilitate monthly sample collection. Three of the selected farms were located in Prince Edward Island and four farms were located in New Brunswick. Farm prevalence ranged from 3% to 15%, and herd size ranged from 83 to 490 cows per herd.

From the seven farms, 51 MAP-infectious Holstein cows were recruited in total, with a range of 2 to 15 cows recruited per farm.

2.3.2 Target and Case Conditions

For this study, the target condition was a MAP-infectious cow that was actively shedding the organism at the time of fecal testing. Cows were considered to be MAP-infectious at the start of the study if they were fecal culture positive (confirmed with acid fast stain and qPCR) a minimum of one time within a one year period prior to the start of this study (case definition), as determined from the companion project. Therefore, for the remainder of this study, the term MAP-infectious is used to identify these cows. Age, parity, and lactation stage information was recorded for each cow at each sampling time.

2.3.3 Sample Collection

All animal protocols were pre-approved by the Animal Care Committee at the University of Prince Edward Island. Individual fecal samples were collected from these 51 MAP-infectious cows using individual, clean rectal sleeves without lubrication, and samples placed into individual, clean plastic specimen jars. Sampling was performed monthly for up to 12 consecutive months (during the period from July 2010 to December 2011) per cow or until removal from the herd. Postpartum fecal samples were collected by the farmers within 14 days of calving. All samples were frozen for long-term storage at -80^{o}C until processing could be completed at the Maritime Quality Milk laboratory (Charlottetown, Prince Edward Island, Canada), which is USDA-accredited for fecal culture and qPCR techniques.

2.3.4 Laboratory Procedures

2.3.4.1 Solid Culture

Solid cultures were performed on HEYM slants supplemented with mycobactin J and amphotericin B, nalidixic acid, and vancomycin (Becton, Dickinson, and Company, Sparks, Maryland), and quantitative CFU counts were performed on positive cultures. Procedures used followed methods described by Stabel (1997). Briefly, 3 g of thawed fecal sample were added to 30 mL of half-strength Brain Heart Infusion (**BHI**) with 0.9% hexadecylpyridinium chloride monohydrate (**HPC**) and vortexed. The suspension was allowed to settle for 30 minutes at room temperature, after which 15 ml of the supernatant was transferred to a 50 ml polypropylene tube, then centrifuged at 1,700 x g for 20 minutes at room temperature. The supernatant was subsequently discarded. The remaining pellet was vortexed with 30 mL of the 0.9% HPC-BHI solution and then incubated overnight at 37˚C. The sample was subsequently centrifuged at 1,700 x *g* for 20 minutes at room temperature, and the supernatant discarded. One hundred μL of 0.9% saline solution was added to the pellet, and the solution vortexed. One hundred μL of this decontaminated, re-suspended sample was inoculated onto the HEYM slant. The tube was incubated for 24 hours horizontally at a slight incline at 37˚C with the cap loose to allow for drying, after which the cap was secured tightly and the tube placed vertically in a rack. The tube was subsequently incubated at 37˚C for 84 days, with weekly examinations under a dissecting microscope for CFU counts until >100 CFU per tube was observed, or until growth of competitive organisms covered the media so that

no MAP colonies were distinguishable. At this point the sample was labelled as contaminated.

2.3.4.2 Broth Culture

Broth cultures were performed using the TREK ESP culture system II (Thermo Scientific, Oakwood Village, Ohio) to obtain quasi-quantitative days to positive counts. This is a pressure detection system in which MAP growth is signaled by decreased pressure in the media bottle. Briefly, 2 g of thawed fecal sample was placed in 35 mL of sterile water and placed on an automatic shaker for 10 minutes followed by 30 minutes of sedimentation at room temperature. Afterwards, 5 mL from the top one-third of the solution was placed in 25 mL of 0.9% HPC-BHI solution and incubated overnight at 37˚C. The following day, the sample was centrifuged at 1,500 x *g* for 20 minutes and the supernatant discarded. One mL of an antibiotic brew consisting of 18.5 mg/mL BHI, 975 µl/mL deionized water, 100 µg/mL vancomycin, 100 µg/mL nalidixic acid, and 50 µg/mL amphotericin B was added to the pellet, vortexed, and again incubated overnight at 37˚C. Subsequently, 2.5 mL of a solution of the kit's Growth Supplement (1 mL), Egg Yolk Supplement (1 mL), and Antibiotic Supplement (0.5 mL) was added to a Para-JEM broth bottle (Thermo Scientific, Nepean, Ontario) along with 1 mL of the decontaminated sample. The bottle was then incubated in the TREK incubator a maximum of 49 days or until a positive pressure signal was detected and confirmed as described below.

2.3.4.3 Direct qPCR

Direct qPCR methods followed the procedures listed for the VetAlert Johne's Real-Time PCR kit (Tetracore, Rockville, Maryland), targeting the *hspX* gene. Briefly, 2 g of thawed fecal sample was added to 35 mL of sterile distilled water, vortexed, placed on an automatic shaker for 15 minutes, and incubated at room temperature for 30 minutes. The top 20 mL of the solution was placed in a new 50 mL centrifuge tube and centrifuged at 2,500 x *g* for 10 minutes at room temperature. The supernatant was discarded, and 1 mL of 1xTE buffer (10 m*M* Tris-HCl, pH 8.0; 1 mM EDTA) was added to re-suspend the pellet.

DNA extraction was then performed as follows: 1 mL of the solution was added to a disruption tube containing sterile glass beads, vortexed, and bead-beat (Mini-Beater 8, BioSpec Products, Bartlesville, Oklahoma) at 4,800 oscillations per minute for 5 minutes. The sample was then centrifuged for 10 minutes at 16,000 x *g*, the supernatant transferred to a new 2 mL microcentrifuge tube, 100 µl Nucleic Acid Buffer (NAB™) Buffer added, and inverted 5 times to mix. The sample was then centrifuged at 1,200 x *g* for 3 minutes, the supernatant discarded, 560 µl of Binding Buffer added to re-suspend the pellet, and incubated at room temperature for 10 minutes. Subsequently, 560 µl of 100% ethanol was added to the tube, which was then vortexed. Part of the sample (630 µl) was added to a spin column placed in a new collection tube and centrifuged at 5,200 x *g* for 1 minute. This same step was repeated with the remaining sample. For all the following steps involving the spin column, a new collection tube was used. Wash Buffer A (500 µl) was added and centrifuged at 5,200 x *g* for 1 minute. Then Wash Buffer B (500 µl) was added and centrifuged at 12,000 x *g* for 3 minutes followed by centrifugation at 16,000 x *g* for 1 minute without buffers. The spin column was then

placed into a microcentrifuge tube, 50 µl of deionized water added, and the sample left at room temperature for 1 minute. The sample was then centrifuged at 5,200 x *g* for 1 minute, and the DNA elution saved at 4˚C for up to 48 hours or otherwise stored at -20˚C.

For the qPCR fluorogenic probe hydrolysis assay procedure, 22.5 µl Master-mix was added to a thermocycler reaction tube. To each sample tube, 2.5 µl of the eluted DNA was also added. To another two tubes, the same amount of positive and negative control was added, respectively. The reaction tubes were then centrifuged for approximately 15 seconds and subsequently loaded into the Cepheid SmartCycler II Thermocycler (Cepheid, Sunnyvale, California). After an enzyme activation step at 95˚C, a two-step cycling reaction at 95˚C and 62˚C was used. Results were recorded as Ct values, with values considered positive if <42 Ct; and the run was considered valid if the positive control values were between 20 and 26 Ct.

2.3.4.4 Culture Confirmation

All broth and solid culture results were confirmed by positive acid fast stain. Any acid fast positive results on either culture method were further confirmed with qPCR. For broth culture confirmation, the bottle was removed from the TREK incubator and placed in an automatic shaker for 5 minutes. One mL from the bottle was then placed in a disruption tube containing sterile glass beads, and the procedures followed for DNA extraction and qPCR test as outlined above under the direct qPCR procedures. For solid culture confirmation, the media tube was removed from the incubator, and 2 or 3 colonies were collected with a sterile loop into a sterile centrifuge tube containing 1 mL

of sterile 0.9% saline and vortexed. One mL of this mixture was then added to the disruption tube containing sterile glass beads, and the DNA extraction and qPCR test procedures as outlined above were followed.

2.3.5 Statistical Analysis

Statistical analysis was performed using STATA/IC Version 12 (StataCorp LP, College Station, Texas, USA). A *P*-value of 0.05 was chosen as a cut-off for statistical significance. Proportions of positive culture results were analyzed overall and at categorized shedding levels. For solid culture, low shedding was set to <10 CFU per culture tube, moderate shedding from 10 to 50 CFU per tube, and high shedding >50 CFU per tube (Crossley et al., 2005). For broth culture, high shedding was set to <21 days to a positive change-in-pressure signal, moderate shedding between 21 and 28 days, and low shedding >28 days (Shin et al., 2000, 2001).

Population averaged cow-level generalized estimating equation logistic models (Dohoo et al., 2009) were used to analyze if season and lactation stage were predictive of solid culture, broth culture, or qPCR positivity. This approach, with an autoregressive (AR1) within-cow correlation structure (for the relationship between times within tests within cows) and robust standard errors was chosen in order to best handle the repeated samples per cow. Separate models were built for each of the three dichotomous outcomes (solid culture, broth culture, qPCR). The model structure was:

$$\text{logit}(p) = \text{intercept} + \text{season} + \text{lactation stage} + \text{farm} + \text{age}$$

where p is the probability for a cow to be test positive relative to the test under evaluation in that model; season is a categorical variable for the season of sample

collection; lactation stage is a categorical variable for the cow's lactation stage at time of sample collection; and farm and age are categorical variables analyzed as possible confounders and referring to the respective farm for and age (years) of the cow at the time of sample collection. Seasons were set to be July through September for summer, October through December for fall, January through March for winter, and April through June for spring to correspond to local weather patterns. Lactation stages analyzed were categorized as follows: <60 days in milk (**DIM**), 60 to 99 DIM, 100 to 239 DIM, ≥240 DIM, and dry. Contrasts and linear comparisons of all significant predictors and any interactions were analyzed using Bonferroni adjustments of *P*-values to account for multiple comparisons.

Agreement between assays was calculated using the following tests: Cohen's Kappa test for agreement beyond chance and McNemar's exact test for differences (significant outcome) between proportions of positive results from the testing methods (Dohoo et al., 2009). Sensitivity, which is the proportion of positive results in the known MAP-positive group of cows, was recorded along with a 95% confidence interval. Conditional Se between tests was calculated, as well as parallel Se, when either or both of the tests being compared were positive (Dohoo et al., 2009).

Finally, the relationship between shedding level from each culture method and qPCR Ct values was also assessed. An additional mixed linear regression model (Dohoo et al., 2009), with random effects at the cow-level, was built using qPCR results for the continuous outcome and using an autoregressive (AR1) within-cow correlation structure (for the relationship between times within tests within cows) and robust standard errors as follows:

$$Y = \text{intercept} + \text{season} + \text{lactation stage} + \text{farm} + \text{age} + u(\text{cow}) + \varepsilon$$

where Y refers to the outcome of the positive (non-zero) qPCR Ct values; the predictors season, lactation stage, farm, and age are explained as for the previous models; and u and ε refer to the random effects. Transformations for the outcome were evaluated, but none were required for maintaining the assumption of linearity for the model. In addition, all relevant interactions for significant predictors were analyzed, as well as pairwise comparisons, using Bonferroni adjustments of *P*-values.

2.4 Results

2.4.1 Descriptive Data

Samples from 51 cows from seven farms were used for this study. Cows ranged from two to nine years of age (mean = 4.5) and from first to seventh parity (mean = 2.9). A total of 395 samples were collected, with 13 of these samples from cows during their 14-day postpartum period and 28 from cows during their dry period (from the time of milking cessation to parturition). Analysis was limited to data from cows with <400 DIM (345 samples), to stay within a typical lactation length and because data were sparse above this threshold. Monthly variation in the detection ability of the three testing methods (solid culture, broth culture, and qPCR) for fecal samples from the MAP-infectious cows is depicted in Appendix A.

2.4.2 Sensitivity of Detection Methods

The Se for each of the three testing methods for the 51 MAP-infectious cows over the full collection, postpartum, and dry periods are listed in Table 2.1. For solid cultures, 32 of the 345 samples were contaminated (9.3% contamination rate), leaving 313 samples available for analysis. For direct qPCR, one sample was lost, leaving 344 samples. Predicted confidence intervals are included for overall data collection, but not for the postpartum and dry periods because sample numbers were too few. For all fecal samples collected, qPCR had a significantly higher Se than either broth or solid culture. Numerically, dry period samples had slightly higher Se than those in other periods, but numbers of samples available from dry cows was very limited.

Shedding levels from the MAP-infectious cows are listed in Table 2.2. Because criteria for each shedding category differ by culture method, comparisons from the data were only made within culture methods rather than between culture methods. As a result, within solid culture results, more cows shedding >50 CFU were identified, but within broth culture results, fewer cows with heavy shedding were identified, as cultures typically required >21 days for a positive signal.

2.4.3 Detection Patterns across Seasons and Lactation Stages

The generalized estimating equation model analyses revealed no significant association between test Se (the probability of an average MAP infectious cow testing positive) and lactation stage with either culture technique. For direct qPCR, lactation stage was significantly associated ($P < 0.01$), especially with the inclusion of the dry period category, in which higher MAP detection was observed. Seasonal patterns in test Se results with direct qPCR were significant as an unconditional association ($P < 0.05$),

yet borderline significant ($P = 0.09$) when considering the confounding effects of lactation stage and farm. There were no statistically significant interactions between season and lactation stage. The probability of the test being positive in the summer and fall seasons was 85% of the probability of the test being positive in the winter and spring seasons. However, after Bonferroni adjustment of *P*-values due to multiple comparisons, there were no significant differences between seasons or lactation stages despite the raw data trends, as some categories had very low sample counts. Predicted probabilities for positive qPCR results within lactation stages by season are shown in Figure 2.1.

For solid culture of MAP, increased failure of the decontamination procedures to eliminate competitive organisms on the media in the summer and fall months, led to more samples labelled as contaminated during those periods. The mean contamination rate was 12.5% within the total of 206 samples from the summer and fall seasons, as compared to 4.2% within 139 samples from the winter and spring seasons. However, the effects of season ($P = 0.22$) and lactation stages ($P = 0.48$) were non-significant for contaminated samples.

2.4.4 Comparison among Detection Methods

The McNemar exact test (Table 2.3) indicated that in all three test comparison combinations, proportions of positive results obtained were significantly different between tests, with qPCR being most sensitive and solid culture being least sensitive. Despite the higher Se of broth over solid culture, Kappa between the two culture methods was approximately twice as high as between qPCR and either culture method. Use of qPCR in parallel testing with culture techniques did not improve Se substantially

over use of qPCR alone. However, conditional Se (data not shown) of qPCR varied from 58.1% if broth culture was negative to 93.7% if broth culture was positive, whereas the Se of broth culture varied from 15.6% to 66.3% if qPCR was negative or positive, respectively.

2.4.5 Patterns in qPCR Values across Shedding Levels

Mean Ct values were also noted according to the corresponding growth pattern in broth or solid media (Table 2.4). For all fecal samples in which either solid or broth culture as well as qPCR results were positive, our study indicated a decreasing trend in qPCR Ct value with increasing MAP shedding. More specifically, on average, a Ct value of <35 cycles was obtained when culture results were positive, with on average a Ct value of <29 cycles corresponding to heavy shedding levels with both culture techniques. In addition, the mean Ct value in summer was 31.3 (95% CI: 30.0 to 32.7) compared to 33.1 (31.9 to 34.2) in fall, 33.9 (32.7 to 35.1) in winter, and 33.8 (32.4 to 35.3) in spring. Pairwise comparisons between seasons indicated that this trend was significant (using Bonferroni adjusted *P*-values) between summer and fall ($P < 0.01$) and between summer and winter ($P = 0.01$). All other season comparisons were non-significant.

2.5 Discussion

Understanding the ability to detect MAP in fecal samples with various testing methods as well as patterns in MAP shedding can lead to efficient testing of animals for enhanced control programs and prioritized management of high MAP-shedding cows.

2.5.1 Sensitivity of Detection Methods

The results of our study support the use of direct fecal qPCR as part of a Johne's herd management program, particularly for known infected herds. Data analysis for pathogen detection revealed a higher Se for qPCR as compared to the Se of broth culture, with an even greater difference from solid culture, for fecal samples from MAP-infectious cows (Table 2.1). This was in contrast to Alinovi et al. (2009), who previously reported qPCR (Tetracore, positive cut-off <38 Ct) Se of 60% and Sp of 97%. However, they reported qPCR accuracy (90%) comparable to the accuracy of solid (HEYM; 91%) and broth (TREK; 93%) culture results. The pattern observed in our data was most apparent in the qPCR results for feces collected during a cow's dry period and post calving period. However, the numbers of observations during these time periods are relatively few, reducing the statistical power of these analyses. Therefore, additional research with larger sample sizes is recommended to confirm the apparent results in these time periods.

2.5.2 Detection Patterns across Seasons and Lactation Stages

An advantage to qPCR that may explain the improved Se is the absence of decontamination steps that could decrease low numbers of bacteria in a sample and hamper detection via culture techniques (Bölske and Herthnek, 2010). Also, qPCR is

able to detect MAP regardless of the presence of competing organisms, as it detects MAP by a different biological principle (genetically) as compared to culture methods.

The presence of competing organisms can occasionally hinder the interpretation of culture results. Generally, a 5 to 15% contamination rate for fecal solid culture is expected (Collins and Manning, 2014). In our data, solid culture of MAP in feces showed a pattern of increased failure of the decontamination procedures to eliminate competitive organisms on the media, leading to more samples potentially being classified as contaminated in summer and fall (12.5%) as compared to winter and spring (4.2%). However, this rate can vary widely depending on the decontamination method used, type of media, and the number of media slants per sample (Whittington, 2010). Our overall contamination rate of 9.3% was within these bounds, as were the seasonal rates. However, the overall low numbers of contaminated samples may have led to the decrease in statistical power to report the seasonal effect for these samples. Using qPCR, particularly in summer and fall, may avoid the need for repetitive cultures to overcome decontamination failures, particularly for the identification of high shedding cows.

In our data, apparent patterns across seasons and lactation stages were also observed for qPCR overall (Figure 2.1). Seasons with more similar climatic temperatures were also more similar in qPCR detection ability. In general, Se was higher in winter and spring, and Se was improved in the dry period. However, due to low numbers of observations within some of the categories for season and particularly lactation stage, statistically significant associations were not observed when accounting for multiple comparisons.

Although we did not find an association between season and culture detection of MAP, the association between season and qPCR detection of MAP agree with other studies looking at the effect of season on MAP growth in culture. Norton et al. (2010) observed higher MAP recovery in spring and indicated a potential correlation between season and lactation stage, in particular seasonal calving. Crossley et al. (2005) reported a higher CFU count (HEYM) during the winter months and in larger herd sizes. The authors of this study suggest stressors that could trigger increased MAP shedding in winter to include adverse weather conditions (Jørgensen, 1977), winter calving, herd management, and body condition. Although this study assessed culture detection of MAP, it is likely that some of these stressors could also explain the patterns seen within our data. However, differing protocols make comparisons between this and other research studies difficult due to varying definitions of herd or cow MAP status, MAP prevalence, test and decontamination protocols, sampling protocols, and herd sizes, among others. Therefore, further research is needed to assess seasonal patterns over a period of several years with more herds, in order to increase statistical power. In addition to this, other important factors to analyze regarding MAP fecal shedding include the impact of housing, dietary changes, and herd management practices that expose youngstock to the manure of adult cattle, from farms of various levels of MAP prevalence and from cows within the dry and postpartum periods of lactation.

2.5.3 Comparison among Detection Methods

The use of qPCR is based on a different biological principle (detection of the organism's genetic material) than culture. Therefore, Pinedo et al. (2008) suggest that

parallel testing with both PCR and serum ELISA resulted in the best detection ability over other combinations of culture, PCR, and serum ELISA assays. Because of this, it was of interest to determine if the higher qPCR Se in our study was based on the detection of a different population of positive animals or better overall detection. To assess this, we examined parallel interpretation of the test with either culture method (Table 2.3). Parallel use did not improve Se substantially over qPCR alone, indicating that qPCR was most sensitive by identifying MAP in additional cows to those detected with culture assays. Kappa also indicated lesser agreement between qPCR and culture, but greater agreement between culture methods.

2.5.4 Patterns in qPCR Values across Shedding Levels

Comparison between solid and broth culture regarding degree of shedding is difficult, as criteria for each shedding category differ by culture method. Because qPCR Se was improved over culture Se in our data, especially in the dry period, it was also of interest to determine if a pattern in Ct ranges could be observed across shedding levels. As a benefit to control programs, the risk of environmental contamination and subsequent infection to calves could be indirectly calculated using qPCR Ct values as an indicator of the degree of fecal MAP shedding in dams without simultaneous culture testing. Our results (Table 2.4) indicated that if the sample yielded a positive broth or solid culture result, qPCR values were typically <35.0 Ct. Moderate and heavy shedding cows, as determined for each culture technique (>10 CFU for solid culture or <28 days to positive for broth culture) typically corresponded to <29.0 Ct.

In a recent study by Leite et al. (2013), a comparison was made between several different qPCR kits and gene sequences for MAP, including the Tetracore kit for both *IS900* and the *hspX* genes. The authors found that qPCR Se and Sp depended on which kit was used and thereby the volume of DNA elution and which gene sequence was identified. For the Tetracore kit identifying the *hspX* gene, they found a Se of 57.1% for samples growing <10 CFU per PCR tube, and that these low shedders on average were detected with a Ct value of 36.2 (range 33.5 to 38.4). Heavy shedding, which they identified with >100 CFU per tube, had a Se of 100% for qPCR with an average Ct of 29.9 (range 26.5 to 35.1). Although in our study we defined a heavy shedder as having >50 CFU per culture tube (Crossley et al., 2005), we did not find any difference in mean Ct value between the 74 observations >50 CFU (26.5 Ct), the 64 observations >100 CFU (26.5 Ct), and the ten observations between 50 and 100 CFU (27.0 Ct).

2.5.5 Conclusion

The primary objectives of our study were to assess the abilities of common fecal testing assays to accurately diagnose MAP-infectious cows and identify any patterns across seasons or lactation stages. Direct fecal qPCR is an invaluable MAP identification test, with higher Se, faster processing times, and lower laboratory costs, as compared to culture methods. Our study highlighted a potential seasonal pattern with increased qPCR Se in winter and spring, as well as an additional benefit to using qPCR in months where culture contamination is more likely (summer and fall). Consideration of Ct values further extends the application of qPCR within MAP control programs, as lower Ct values are indicative of greater MAP bacterial load in a cow's feces. This allows for

prioritized management of high shedding cows, and could ultimately increase the success of herd MAP control programs.

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Table 2.1. Sensitivity (%) of solid culture (Herrold's egg yolk media with mycobactin J), broth culture (TREK ESP system; Thermo Scientific, Ohio), and real-time PCR (qPCR; VetAlert; Tetracore, Maryland) for fecal samples collected monthly over a 12 month period from 51 Mycobacterium avium subsp. paratuberculosis infectious cows from seven herds.

	Solid culture	Broth culture	qPCR
All samples	45.7 (40.1-51.2[a]) [313[b]]	54.8 (49.5-60.1) [345]	77.6 (73.2-82.0) [344]
Postpartum[c]	33.3 [18]	47.6 [21]	81.0 [21]
Dry period	60.0 [11]	81.8 [11]	90.9 [11]

[a]95% confidence interval, listed if more than 40 observations available.
[b]Number of observations.
[c]Feces collected up to 14 days post-calving.

Table 2.2. Proportion (%) of solid culture (Herrold's egg yolk media with mycobactin J) and broth culture (TREK ESP system; Thermo Scientific, Ohio) outcomes across shedding levels for fecal samples collected monthly over a 12 month period from 51 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

	Shedding Levels			
	Negative	Low	Moderate	Heavy
Solid culture[a]	54.3 (170[c])	15.0 (47)	7.0 (22)	23.6 (74)
Broth culture[b]	45.2 (156)	29.9 (103)	9.0 (31)	15.9 (55)

[a]Solid culture: low (<10 CFU per culture tube), moderate (10 to 50 CFU), high (>50 CFU) (Crossley et al., 2005).
[b]Broth culture: low (>28 days to a positive signal), moderate (21 to 28 days), high (<21 days) (Shin et al., 2000; Shin et al., 2001).
[c]Number of observations at the corresponding shedding level.

Table 2.3. Individual and parallel sensitivity comparisons and Cohen's Kappa agreement between solid culture (Herrold's egg yolk media with mycobactin J), broth culture (TREK ESP system; Thermo Scientific, Ohio), and real-time PCR (qPCR; VetAlert; Tetracore, Maryland) for fecal samples collected monthly over a 12 month period from 51 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

Test		N[c]	Sensitivity (%)			Kappa
1	2		Test 1	Test2	Parallel	
Solid	Broth	313	45.7[a]	56.9[b]	59.1	0.691
qPCR	Solid	312	78.8[a]	45.8[b]	80.1	0.321
qPCR	Broth	344	77.6[a]	54.9[b]	81.1	0.373

[a-b]Sensitivities within a row with different superscripts differ ($P < 0.01$) using the McNemar exact test.
[c]Number of observations when results from both tests were available concurrently.

Table 2.4. Mean real-time PCR (qPCR; VetAlert; Tetracore, Maryland) cycle threshold values corresponding to bacterial growth patterns in solid culture (Herrold's egg yolk media with mycobactin J) or broth culture (TREK ESP system; Thermo Scientific, Ohio) for fecal samples collected monthly over a 12 month period from 51 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

	Shedding levels			
	Negative	Low	Moderate	High
Solid[a]	36.4 (30.0-42.7[c]) [107[d]]	34.5 (28.8-40.2) [44]	32.1 (27.1-37.1) [21]	26.5 (18.9-34.2) [74]
Broth[b]	36.8 (29.7-43.9) [90]	34.3 (28.8-39.9) [94]	29.2 (20.7-37.7) [30]	26.6 (17.3-36.0) [53]

[a]Solid culture: low (<10 CFU per culture tube), moderate (10 to 50 CFU), high (>50 CFU) (Crossley et al., 2005).
[b]Broth culture: low (>28 days to a positive signal), moderate (21 to 28 days), high (<21 days) (Shin et al., 2000; Shin et al., 2001).
[c]95% confidence interval.
[d]Number of observations.

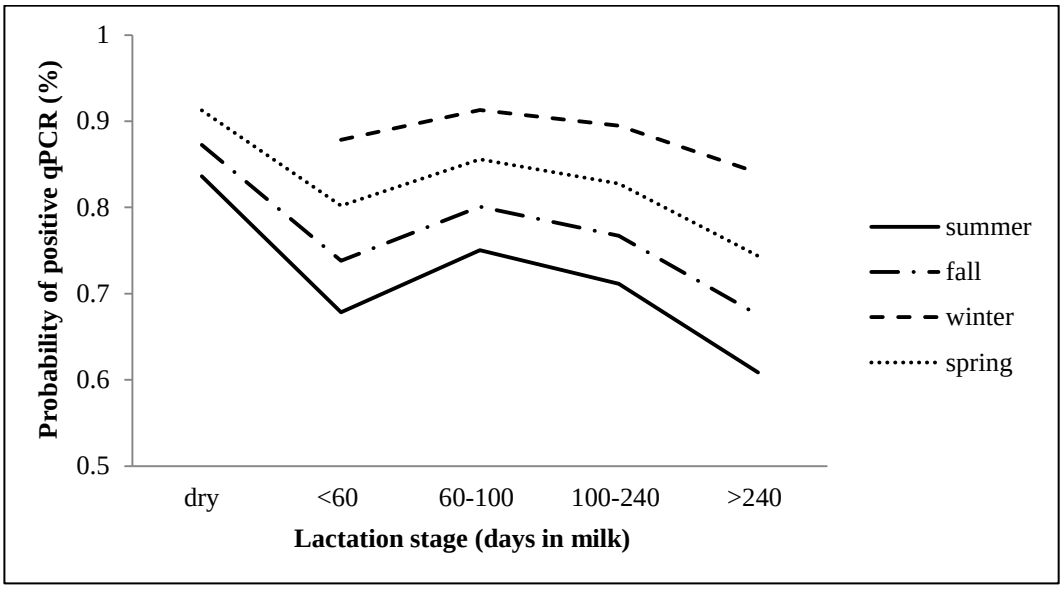

Figure 2.1. Predicted probability (%) for positive direct real-time PCR (qPCR; VetAlert; Tetracore, Maryland) across lactation stage categories by season for fecal samples collected monthly over a 12 month period from 51 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

CHAPTER 3

DETECTION ABILITY OF SOLID CULTURE, BROTH CULTURE, AND REAL-TIME POLYMERASE CHAIN REACTION ASSAYS FOR MILK AND COLOSTRUM SAMPLES FROM *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* INFECTIOUS DAIRY COWS

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3.1 Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) can be shed not only in feces but also secreted in milk and colostrum. The goal of this study was to assess detection of MAP in milk and colostrum and any effect of lactation stage or season on the detection of MAP-shedding. This knowledge can improve our use of both culture and molecular methods for identification of MAP to assess risk of infection to calves. Forty-six previously confirmed MAP-positive cows from seven Atlantic Canadian dairy farms were identified for monthly sampling over a 12 month period. Samples were analyzed with solid culture, broth culture, and real-time PCR (qPCR). For any method used for MAP detection in milk or feces, the detection capacity for milk samples was on average 25% that for fecal samples. However, for colostrum, MAP detection ability depended on the method utilized. With qPCR, MAP detection ability within colostrum was approximately 46% of detection within feces. For both milk and colostrum, higher detection ability was observed with qPCR than with either culture method. Seasonal effects were observed for qPCR results in milk samples, with the highest sensitivity in summer. Summer was also the season when there was strongest agreement between milk and fecal samples collected within the same month. Although MAP presence was identified in milk and colostrum samples with all three pathogen detection methods, improved detection with direct qPCR could lead to more efficient identification of

MAP-infectious cows shedding the bacteria in milk and colostrum and, thereby, provide management information to help decrease the risk of exposure to calves.

3.2 Introduction

Johne's disease, or paratuberculosis, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (**MAP**), and is an incurable, production-limiting disease of dairy cattle. Although it manifests as chronic diarrhea in adult cows (affected cows), it has a characteristically long preclinical stage (infected cows), ranging from 2 to 10 years, during which infected cows can intermittently shed detectable bacteria (infectious cows) (Whittington and Sergeant, 2001; Nielsen and Toft, 2008). In addition to fecal shedding, MAP can also be shed via the mammary gland in milk and colostrum (Lombard, 2011). Calves are the most susceptible to infection, with the highest risk in calves less than one month of age (Sweeney, 2011). Calves that have not become infected *in utero* are most likely to become infected after birth by ingesting MAP through directly infected or fecal-contaminated colostrum or milk, or by ingesting MAP-contaminated feces from the udder or environment (Sweeney, 2011). Affected cows and heavy-shedding infectious cows can shed large amounts of MAP in their milk (Sweeney et al., 1992; Whittington and Sergeant, 2001; Rademaker et al., 2007). A calf risks infection by ingesting as little as 50 colony forming units (**CFU**) of MAP (Chiodini, 1996). In a study by Streeter et al. (1995) of preclinical cows shedding MAP in their feces, three times as many of these cows had the bacterium isolated from their colostrum than from milk, and

approximately 22% of these preclinical cows were found to be shedding MAP in colostrum. Detection ability varies due to factors such as infection stage, assay type, and laboratory methods. A recent meta-analysis of MAP shedding through milk, as detected with culture and polymerase chain reaction (**PCR**), found an overall apparent MAP prevalence of 20% in individual milk samples (Okura et al., 2012). Stressful situations, including parturition, peak milk production, and environmental changes, can generate or increase clinical signs and subsequent MAP shedding (Sweeney, 2011; Bradner et al., 2013a). In a literature review of the influence of stress on bacteria in animals, stress-related hormones, particularly catecholamines, released from the sympathetic nervous system, and glucocorticoids, released from the hypothalamic-pituitary-adrenal axis, have been shown to affect not only the host's immunity but also the bacteria directly (Dhabhar, 2009; Verbrugghe et al., 2012). In particular, these stress-hormones can down-regulate cell-mediated cytokines and immunity and up-regulate humoral immunity through directly influencing host-pathogen interactions (such as at enteromucosal sites), the growth and virulence of pathogens, and cytokine production and expression (Elenkov and Chrousos, 1999; Lyte, 2004; Verbrugghe et al., 2012). For MAP, the direct and chronic effect of stress-hormones on the macrophage-pathogen interplay can negatively impact disease resistance in the host and enhance disease progression and bacterial shedding (Verbrugghe et al., 2012).

In much of the literature, culture has been used as the reference standard for direct bacterial identification (Bölske and Herthnek, 2010). Unfortunately, the slow growth rate of MAP requires culture incubation times of up to seven weeks on broth media and 16 weeks on solid media (Whittington, 2010). However, as MAP bacterial

numbers inherently tend to be much lower in milk and colostrum than in feces, additional sedimentation, centrifugation, and filtration steps can improve bacterial concentration, but subsequent decontamination with incubation in an antimicrobial brew can decrease viable bacterial load in the sample. In an effort to minimize this bacterial loss, it is now generally accepted that both the cream and pellet portions should be incorporated in the pre-culture steps, or even the DNA extraction steps, following centrifugation, for culture methods or molecular methods, respectively (Gao et al., 2005; Gao et al., 2009; Bradner et al., 2013a).

Molecular testing via PCR methods has been developed for MAP detection directly or as culture confirmation. Real-time PCR (**qPCR**) is a rapid technique used to detect both viable and non-viable MAP compared to culture, and takes less than 48 hours to obtain results. In addition, no decontamination techniques are required prior to DNA extraction (Bölske and Herthnek, 2010). Although the insertion element *IS900* is the usual sequence targeted, other gene elements detected in milk and colostrum include *F57* and *ISMAP02* (Pithua et al., 2011a; Hanifian et al., 2013). The sequence *hspX* is another sequence that is unique to MAP (Ellingson et al., 1998; Bölske and Herthnek, 2010) and is used in the Tetracore Real-time qPCR kit for Johne's detection (Tetracore, Rockville, Maryland).

The incurable nature and insidious preclinical phase of paratuberculosis require adequate identification of infected and infectious animals if a producer wishes to increase biosecurity, decrease transmission into and within the herd and, most importantly, decrease exposure of calves (Whitlock, 2010). Control of fecal contamination of the environment, particularly areas specified for parturient and dry

cows, and segregation of high-shedding cows becomes a priority (Pithua et al., 2013). Another priority involves feeding calves colostrum or waste milk that does not contain the organism (Diéguez et al., 2008; Pithua et al., 2011b). Pooled colostrum and colostrum or waste milk from MAP-infected cows should not be used. Rather, milk replacer, properly pasteurized waste milk, or milk from a single non-infected dam is recommended (Nielsen et al., 2008; Garry, 2011; Lombard, 2011). Despite these widely endorsed recommendations, there is conflicting evidence about their efficacy. In a recent study, MAP exposure via colostrum had no effect on subsequent MAP infection, although the authors advise that these results be cautiously interpreted (Pithua et al., 2011a). Another study found that in an infected herd, more than 81% of the MAP-positive colostrum or MAP-positive teat swabs came from potential environmental seeding of MAP rather than direct shedding (Pithua et al., 2011b).

Because MAP has been identified from patients with Crohn's disease, public health concerns of a zoonotic link between MAP and Crohn's disease, a chronic enteritis in humans, spur ongoing research. The concerns also involve the possibility of milk and milk products as a vehicle for transmission to humans, particularly as research has shown that, to a certain extent, MAP can survive commercial high-temperature, short-time pasteurization techniques for milk (Grant et al., 2002; Manning and Collins, 2010; Van Brandt et al., 2011). Of great concern are the recent findings of MAP presence in baby formulas. With both *IS900* and *F57* qPCR, Hruska et al. (2005, 2011) have reported MAP concentrations as high as 32,500 cells/g of some commercial baby formulas.

For producers, the ability of MAP to survive some pasteurization techniques also presents a further concern. Some producers rely on on-farm pasteurized colostrum and milk for their calves. Although most MAP can be killed in colostrum at 60˚C for 60 minutes (a recommended protocol for on-farm pasteurization), the results are still variable and depend on the MAP load within the sample (Godden et al., 2006).

Seasonal effects on MAP detection in pasteurized milk samples for retail sales have been reported (Ellingson et al., 2005): a British study looked at MAP transmission into retail milk and found increased MAP detection in samples between December and March (Millar et al., 1996; Grant et al., 2002); but limited information is available regarding effects of stage of lactation and season on MAP detection in milk and colostrum samples. In a study on bulk tank milk, MAP concentration was found to be potentially related to seasonal calving and stage of Johne's disease (Cazer et al., 2013). As well, Bradner et al. (2013b) and Stabel et al. (2014) detected higher MAP shedding in colostrum and milk in more advanced disease stages and in early lactation periods.

Understanding patterns of MAP shedding, not just in feces but also in milk and colostrum, can support development of more time-efficient and cost-efficient detection strategies. The first objective of our study was to assess MAP pathogen detection in milk and colostrum with three assays (solid culture, broth culture, and qPCR). In order to do so, MAP detection ability in milk and colostrum was assessed across assay-specific shedding categories, and detection ability within milk and colostrum samples was compared to that of fecal detection methods in previously determined MAP-infectious cows. A second objective was to identify any effects of season or lactation stage on MAP detection within milk and colostrum samples from the same group of MAP-

infectious cows. This knowledge is important for enhancing the efficient use of diagnostic tests for MAP infection in milk and colostrum. Improved recommendations for control and monitoring of this disease within a herd can thereby decrease the risk of exposure and subsequent infection in calves.

3.3 Materials and Methods

3.3.1 Farm and Cow Selection

From a companion project assessing MAP herd diagnostics in Atlantic Canadian dairy herds (Lavers et al., 2013), we purposively selected seven dairy farms: three from Prince Edward Island and four from New Brunswick, Canada.Within-herd MAP prevalence (as determined by pooled fecal culture, followed by individual fecal culture of positive pools and confirmed with acid fast staining and qPCR) in these MAP-positive herds ranged from 3% to 15% in the previous year (C. Lavers; Atlantic Veterinary College, Charlottetown, PE, Canada, personal communication). Herd sizes ranged from 83 to 490 cows per herd.

From these herds, a total of 46 MAP-infectious Holstein cows, actively shedding detectable MAP in their feces (target condition), were recruited, ranging between 2 to 15 cows recruited per farm. A cow was labelled MAP-infectious if it was fecal culture positive (confirmed with acid fast stain and qPCR) at least once during a one year period prior to the start of the current study (case definition). At each sampling period, age, parity, and days in milk were also recorded for each cow.

3.3.2 Sample Collection

Protocols were approved by the Animal Care Committee at the University of Prince Edward Island prior to the study. Fecal samples from each cow were collected monthly with clean, individual rectal sleeves, without lubrication, for up to 12 months (from July 2010 to December 2011), as long as the cow remained in the herd. From the 46 MAP-infectious cows, 36 cows had colostrum samples collected by the farmer within 24 hours of parturition. Clean milk samples were collected monthly either by the project personnel or by the farmers (these samples were subsequently stored on-farm at -20˚C), and all samples were transported, monthly, on ice to the Maritime Quality Milk Laboratory at the University of Prince Edward Island, Canada, where they were frozen at -80˚C until processing. The Maritime Quality Milk Laboratory is USDA proficiency tested for fecal culture and qPCR.

3.3.3 Laboratory Procedures

3.3.3.1 Fecal Solid Culture

Solid cultures were performed on Herrold's egg-yolk media (**HEYM**) slants supplemented with mycobactin J and amphotericin B, naladixic acid, and vancomycin (Becton, Dickinson, and Company, Sparks, Maryland). Procedures followed, in part, methods described by Stabel (1997). Briefly, 3 g of thawed fecal sample was added to 30 ml of half-strength Brain Heart Infusion (**BHI**) with 0.9% hexadecylpyridinium chloride monohydrate (**HPC**; Sigma Chemical Company, St. Louis, MO). After sitting at room temperature for 30 minutes, 15 ml supernatant was transferred to a new 50 ml

polypropylene tube and centrifuged at 1,700 x *g* for 20 minutes at room temperature. The supernatant was then discarded. To the remaining pellet, 30 ml of the 0.9% HPC-BHI solution was added, and the suspension was incubated overnight at 37˚C. The next day, the suspension was centrifuged at 1,700 x *g* (IEC CL31 Multispeed Centrifuge, Thermo Scientific, Oakwood Village, Ohio) for 20 minutes at room temperature, and the supernatant was subsequently discarded. To the remaining pellet, 100 µl of 0.9% saline solution was added. Then, 100 µl of the re-suspended sample was inoculated onto the HEYM slant, which was placed at a slight incline at 37˚C. After the inoculation dried, the cap was secured tightly and the tube placed vertically at 37˚C for 84 days. Cultures were examined weekly with the aid of a dissecting microscope, and CFU recorded until >100 CFU/tube was observed, or until growth of competitive bacteria and fungi covered any detectable MAP colonies. If this occurred, the sample was labelled as contaminated. All culture positive results were confirmed with acid fast stain and qPCR as described below.

3.3.3.2 Milk and Colostrum Solid Culture

Procedures followed methods described by Donaghy et al. (2008) for milk, by Godden et al. (2006) for colostrum. Because culture decontamination techniques can decrease MAP concentration, and MAP concentration was expected to be lower in milk and colostrum than feces, we followed the protocol described below, which includes modifications as a result of consultation with Dr. Mutharia (Ontario Veterinary College, Guelph, Ontario, personal communication), and Dr. DeBuck (University of Calgary, Alberta, personal communication). In brief, 30 ml of milk sample or colostrum sample

was centrifuged at 2,800 x *g* for 30 minutes at room temperature (21˚C), and the whey fraction discarded. The cream and pellet fractions were re-suspended completely in 30 ml of 0.75% HPC. Following incubation at room temperature for 5 hours and re-centrifugation at 2,800 x *g* for 30 minutes at room temperature, the pellet was diluted in 100 µl of a 0.9% saline solution and vortexed to re-suspend. From this sample, 100 µl was inoculated onto the HEYM slant, placed at a slight incline at 37˚C until dry, after which the cap was secured tightly and the tube placed vertically at 37˚C for 84 days. Weekly examinations were performed, with the aid of a dissecting microscope, and CFU was recorded until >100 CFU/tube was observed, or until growth of competitive organisms covered any detectable MAP colonies. If this occurred, the sample was labelled as contaminated. All culture positive results were confirmed with acid fast stain and qPCR, as described below.

3.3.3.3 Fecal Broth Culture

The ESP culture system II (TREK Diagnostic Systems, Thermo Scientific, Oakwood Village, Ohio) was used in order to obtain quasi-quantitative days-to-positive counts. In this system, MAP growth is signaled by decreased pressure detected in the head space of the media bottle. As per the kit's instructions, 2 g of thawed fecal sample in 35 ml of sterile water was shaken for 10 minutes on an automatic shaker (Mistrel Multi-Mixer 4600, Barnstead Lab-Line, Melrose Park, Illinois) and then allowed to settle for 30 minutes at room temperature. Five ml from the top one-third of the solution was then added to 25 ml of 0.9% HPC-BHI solution. This sample was incubated overnight at 36˚C, and then centrifuged at 1,500 x *g* for 20 minutes. One ml of an

antibiotic brew (18.5 mg/ml BHI, 975 µl/ml deionized water, 100 µg/ml vancomycin, 100 µg/ml nalidixic acid, and 50 µg/ml amphotericin B) was added to the pellet and re-suspended by vortexing, then incubated overnight at 36˚C. The following day, 2.5 ml of a solution comprising 1 ml of ESP Growth Supplement, 1 ml Egg Yolk Supplement, and 0.5 ml Antibiotic Supplement was added to a Para-JEM broth mycobottle (Thermo Scientific, Nepean, ON, Canada), followed by the addition of 1 ml of the decontaminated sample. The culture was then incubated in the TREK incubator until growth was signaled, up to a maximum of 49 days or until a positive pressure signal was detected. All samples incubated to 49 days, and all positive samples were confirmed with acid fast stain. Any positive results on either broth culture or acid fast stain were confirmed with qPCR as described below.

3.3.3.4 Milk and Colostrum Broth Culture

Briefly, 30 ml of the milk or colostrum sample was centrifuged at 2,800 x *g* for 30 minutes. The whey was decanted, and to the remaining cream and pellet, 30 ml of 0.75% HPC was added. The suspension was then incubated for 4 hours at room temperature. Afterwards, the sample was centrifuged at 1,500 x *g* for 20 minutes and followed the remaining procedure for fecal broth culture described above.

3.3.3.5 Fecal Direct qPCR

Methods followed the procedures listed for the VetAlert Johne's Real-Time PCR kit (Tetracore, Rockville, Maryland), targeting the *hspX* gene. Briefly, 2 g of thawed fecal sample was vortexed in 35 ml of DNase free water and shaken for 15 minutes on

an automatic shaker. The sample was then incubated for 30 minutes at room temperature. The top 20 ml of the solution was centrifuged at 2,500 x *g* for 10 minutes at room temperature. One ml of 1xTE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) was used to re-suspend the pellet.

DNA extraction was then performed as follows: 1 ml of the solution was added to a cell-disruption tube containing sterile glass beads and bead-beat (Mini-Beater 8, BioSpec Products, Bartlesville, Oklahoma) at 4,800 oscillations per minute for 5 minutes, and then centrifuged for 10 minutes at 16,000 x *g* (IEC MicroCL 21R Microcentrifuge, Thermo Scientific, Oakwood Village, Ohio). To the decanted supernatant 100 µl Nucleic Acid (NAB™) Buffer was added, and it was then centrifuged at 1,200 x *g* for 3 minutes. The remaining pellet was re-suspended with 560 µl of Binding Buffer and incubated for 10 minutes at room temperature. Then, 560 µl of 100% ethanol was added, after which 630 µl of the sample was placed in a spin column and centrifuged at 5,200 x *g* for 1 minute. The remaining sample was added to the spin column and centrifuged at 5,200 x *g* for 1 minute. Next, 500 µl of Wash Buffer A was added to the spin column, which was then centrifuged at 5,200 x *g* for 1 minute, followed by the addition of 500 µl of Wash Buffer B to the spin column and centrifugation at 12,000 x *g* for 3 minutes and again at 16,000 x g for 1 minute without additional buffers. The spin column was placed into a microcentrifuge tube, 50 µl of deionized water was added, followed by 1 minute incubation at room temperature, and then centrifugation at 5,200 x *g* for 1 minute. The DNA elution was saved at 4˚C for up to 48 hours or at -20˚C for longer storage.

The qPCR procedure was a fluorogenic probe hydrolysis assay in which 22.5 µl thawed master-mix and 2.5 µl of the eluted DNA was added to a thermocycler reaction tube. For quality control, positive and negative control samples were included in each run of qPCR. The reaction tubes were centrifuged for approximately 15 seconds in a Cepheid microcentrifuge before being placed in the Cepheid SmartCycler II Thermocycler (Cepheid, Sunnyvale, California). After an enzyme activation step (95˚C), a two-step cycling reaction (95˚C and 62˚C) was used. Cut-off value for the positive control was set to between 20 to 26 cycle threshold values. Samples that showed positive within 42 cycles were called MAP-positive.

3.3.3.6 Milk and Colostrum Direct qPCR

Direct qPCR procedures followed a modified version of the method described by Gao et al. (2007). For milk, 30 ml of sample was heated in a water bath (95˚C) for 10 minutes then cooled in ice water for 10 minutes. The milk sample was then centrifuged at 2,800 x *g* for 30 minutes at room temperature. The pellet and cream was re-suspended in 6 ml of 0.75% HPC and incubated for 30 minutes at room temperature. The sample was then centrifuged at 2,000 x *g* for 15 minutes at room temperature. The liquid phase and cream were decanted. The pellet was transferred to a 2 ml microcentrifuge tube, and 1 ml TRIS (pH 8) and 20 µl protein kinase were added. The sample was transferred to a cell-disruption tube containing sterile glass beads and homogenized in the bead beater (Mini-Beater 8, BioSpec Products, Bartlesville, Oklahoma) at 4,800 oscillations per minute for 3 minutes, incubated in a water bath for 10 minutes at 56˚C, and then re-

homogenized in the bead beater as before and cooled on ice. The steps to complete the extraction and qPCR reaction were performed as described above for fecal direct qPCR. For quality control, positive and negative control samples were also included in each run of qPCR. In order to limit the risk of false negative samples, an inhibition control was also added.

Briefly for colostrum, 30 ml of sample was centrifuged at 2,800 x *g* for 30 minutes at room temperature, and the whey and cream decanted. The pellet was transferred to a 2 ml microcentrifuge tube, and 1 ml TRIS (pH 8) was added. The sample was transferred to a cell-disruption tube containing sterile glass beads and homogenized in the bead beater (Mini-Beater 8, BioSpec Products, Bartlesville, Oklahoma) at 4,800 oscillations per minute for 5 minutes. The further steps to complete the extraction and qPCR reaction were performed as described above for fecal direct qPCR. For quality control, positive and negative control samples were also included in each run of qPCR. In order to limit the risk of false negative samples, an inhibition control was also added.

3.3.3.7 Culture Confirmation

All broth and solid culture results were confirmed with acid fast stain, and any positive results on either culture or acid fast stain were further confirmed with qPCR. For broth culture confirmation, the bottle was placed in an automatic shaker for 5 minutes. One ml of the broth culture was added to a cell-disruption tube containing sterile glass beads. The procedure then followed the DNA extraction and qPCR methods described above.

For solid culture confirmation, 2 or 3 colonies were collected from the media with a sterile loop and mixed with 1 ml of sterile 0.9% saline in a sterile centrifuge tube. One ml of the solution was then placed in a cell-disruption tube containing sterile glass beads, and again the DNA extraction and qPCR test procedures, as described above, were followed.

3.3.4 Statistical Analysis

Statistical analysis for test results was performed using STATA/IC 12 (StataCorp LP, College Station, Texas, USA), and statistical significance was set at a *P*-value of <0.05. Sensitivity (**Se**; proportion of positive results in previously determined MAP-infectious cows) was recorded along with a 95% confidence interval (**CI**) for each testing method for all sample types over assay-specific shedding levels. For solid culture, low shedding was established as <10 CFU per culture tube, moderate shedding from 10 to 50 CFU per tube, and high shedding at >50 CFU per tube (Crossley et al., 2005). For broth culture, high shedding was described as <21 days-to-positive, moderate shedding between 21 and 28 days, and low shedding between 29 and 49 days (Shin et al., 2000, 2001). Proportions of detectable MAP shedding in milk when there was concurrent fecal shedding per month were further assessed.

Population averaged, cow-level, generalized estimating equation models were used for the milk analysis. This model structure, with an autoregressive (AR1) within-cow correlation structure (for the relationship between times within tests within cows) and robust standard errors, best handled the repeated samples per cow due to the chronic nature of the disease, intermittent shedding of the bacteria, and possible disagreement

between fecal and milk shedding of the bacteria. Separate models were run for each of the dichotomous outcomes of solid culture, broth culture, and qPCR. Season (categorical) and lactation stage (categorical) were the main predictors of interest. Other predictors included farm (categorical) and age (categorical; years) to assess for confounding. Seasons were categorized as follows: July through September for summer, October through December for fall, January through March for winter, and April through June for spring. Lactation stages were divided as follows: <60 days in milk (**DIM**), 60 to 99 DIM, 100 to 239 DIM, and ≥240 DIM.

Because there were no repeated samples, colostrum results were analyzed with simple logistic models (one for each of the 3 dichotomous outcomes of solid culture, broth culture, and qPCR), including the main categorical predictor of interest for season, and assessing any other potential categorical predictors for farm, age, or parity. Any predictors with *P*-values <0.2 in univariable models were further analyzed in multivariable models.

3.4 Results

3.4.1 Descriptive Data

Cows ranged in age from two to eight years (median = four years) and from first to seventh parity (median = second). Because data were sparse above 400 DIM and in order to stay within a typical lactation length, analyses were limited to results below this threshold, resulting in 298 fecal samples and 304 milk samples for analysis. Thirty-seven colostrum samples were collected from unique parturitions from 36 cows. There

were 296 occasions when milk and fecal samples were collected within the same months, and 37 occasions when colostrum and fecal samples were collected within the same months.

For the fecal samples, 18 of 298 solid cultures were contaminated with overgrown fungal organisms (6.0% contamination rate), while for milk samples, 13 of 304 solid cultures were contaminated (4.3% contamination rate). Contaminated solid cultures were removed from the analyses. In addition to these samples, one fecal sample for qPCR analysis was lost, and 31 milk samples and six colostrum samples had insufficient volume for testing with all three assays.

Of the 46 cows in our case group, only 1 cow never shed detectable MAP in its feces during our study period, and this cow was culled after only 1 sampling. There was detectable fecal MAP shedding in 14 of these cows between 25% and 88% of their sampling times. Furthermore, of the same 46 cows in our case group, 10 cows never shed detectable MAP in their milk during the study period. On the other hand, 1 cow, with no detectable MAP in milk or feces at 1 sampling prior to detectable MAP-shedding in colostrum, did have detectable shedding in both milk and feces during the sampling times after calving.

Monthly variation in the detection ability of the three testing methods (solid culture, broth culture, and qPCR) for fecal and milk samples from the MAP-infectious cows is depicted in Appendix A.

3.4.2 Detection Ability of Assays

Using history of MAP shedding in the previous year to establish the reference standard, overall Se values for each of the three testing methods within all samples are listed in Table 3.1. Predicted 95% CIs were included when there were more than 40 observations per test available. There was superior detection ability for fecal samples, as compared to milk and colostrum samples. For all sample types, qPCR had the highest Se followed by broth culture and then solid culture.

The shedding classification of sample outcome by solid culture (Crossley et al., 2005) and by broth culture (Shin et al., 2000, 2001) for each sample type is presented in Table 3.2. Using broth cultures, high shedding cows were identified with neither colostrum nor milk sampling. Using solid culture results, high shedding cows were identified with both milk and colostrum samples.

3.4.3 Concurrent Milk and Fecal Detection Patterns

Table 3.3 shows further comparisons regarding detection of MAP in both milk and feces collected from the same cow during the same month. When MAP was detected in feces with any of the three pathogen detection methods, 36.5% of these cows were also found to be shedding MAP in their milk, with at least one of the three assays used. Using qPCR detection of MAP in milk, an average of 22.7% of those concurrently shedding MAP in feces was identified. Furthermore, 6.4% (95% CI: 2.3% to 10.4%) and 9.2% (95% CI: 4.0% to 14.4%) of negative fecal solid and broth culture results, respectively, were identified as low shedding in milk, using the same culture method.

3.4.4 Seasonal and Lactational Patterns in Detection Ability

The statistical models for milk and colostrum showed no significant associations between season and detection of MAP in colostrum with any of the three assays. However, power was limited by sample size. There was also no significant association between lactation stages and detection of MAP in milk with all three assays, although model analyses for direct qPCR results for milk samples showed a significant association with the categorical predictor for season ($P < 0.005$), with farm included as a potential confounding variable. Predicted probabilities (including predicted CI) for positive qPCR results within seasons are shown in Figure 3.1. Contrasts with Bonferroni adjustments for *P*-values due to multiple comparisons confirmed a significant difference between summer and fall ($P < 0.005$) and between summer and winter ($P = 0.02$). Contrasts were borderline significant between summer and spring ($P = 0.07$) and between winter and fall ($P = 0.06$). The probability of obtaining a positive qPCR result in milk samples during the fall, winter, and spring seasons was only 43.7% of the probability of the test being positive in summer.

For culture of MAP in milk samples on solid media, there was a noticeable pattern of increased failure of the decontamination procedures to eliminate competitive organisms on the media (13 contaminated samples), leading to more samples being classified as contaminated in the summer (six samples from 65 total samples in the summer) and fall months (five samples from 77 samples), than in the winter (one sample from 45 samples) or spring (one sample from 57 samples).

As season was a significant predictor of MAP shedding in milk, the least amount of MAP shedding in milk was detected during the fall season, when there was

concurrent detectable fecal shedding. In contrast, the best agreement between milk and fecal shedding was seen during the summer season.

3.5 Discussion

Johne's disease is one of the animal health priorities in the Canadian dairy industry. The disease has worldwide distribution (Collins, 2003; Singh et al., 2013) and was listed as having serious economic and zoonotic concerns by the Office International Des Epizooties in 2004 (OIE, 2004). The greatest animal health concern of MAP-infected milk and colostrum is an increased transmission of the disease to calves, which are at highest risk for infection (Sweeney, 2011). There is also a public health concern related to the possibility of MAP seeding into the milk supply (Sweeney, 2011; Van Brandt et al., 2011) These concerns have driven more research into efficient and effective methods of detection of the bacteria in milk and colostrum samples.

3.5.1 Detection Ability of Assays

Our study on the ability of the three pathogen detection methods to identify MAP had two main objectives. First, we looked at the overall detection abilities of the three assays to identify our target condition of MAP-infectious cows (Table 3.1). Second, the results within milk and colostrum samples were compared to the results obtained for concurrent fecal samples. However, comparisons of solid and broth culture outcomes

should be cautiously interpreted, because the assay specific shedding classifications may not be equivalent (Table 3.2).

This study found that regardless of the assay chosen, when the same method was used for MAP detection in both milk and fecal samples, MAP detection ability within milk was, on average, 25% as effective as fecal detection methods. In contrast, MAP detection ability within colostrum was dependent on which assay was used. Using broth culture, detection ability within colostrum was approximately 19% of that within feces, as compared to 6% using solid culture and 46% using qPCR. It should be noted that sample size in the colostrum data is small, and results should be interpreted with caution.

Overall, for all three sample types, qPCR identified the greatest proportion of MAP-infectious cows within our study group (Table 3.1). It is possible that low numbers of bacteria in the milk or colostrum samples or loss of viable cells via decontamination techniques may have led to no growth on culture media and, thereby, false negatives for low-shedding animals that could still be detected by qPCR. Culture of MAP in milk and colostrum can be difficult due to chemical inhibitors in the sample, the presence of low numbers of bacterial cells within the sample, or the clumping of bacterial cells (Gao et al., 2005; Pinedo et al., 2008). Detection by culture can also be hindered via loss of bacterial cells either through centrifugation, where cells may be fractionated into the whey portion, or through decontamination, where MAP bacteria may be killed (Gao et al., 2005; Pinedo et al., 2008). Therefore, our study supports the use of qPCR in Johne's control programs.

Exact comparisons of the Se of the pathogen detection assays for milk, colostrum, and feces between our study and other studies are difficult due to variations

in methodologies. These variations included culture techniques, media, PCR methods, and selection of the target gene. In addition, the disease stage of cows within study groups could affect the degree of bacterial shedding into milk or colostrum. For example, in recent studies in which milk samples from clinical cows were tested using a different liquid culture and decontamination protocol, MAP detection Se was approximately 9% with solid (HEYM) culture, 11% with broth (Bactec 12B) culture, and 39% with PCR (using *IS900* gene target) (Bradner et al., 2012; Bradner et al., 2013b). These culture Se values were substantially lower than those from our study. In contrast, a study by Gao et al. (2009) of 146 cows from 14 MAP-positive herds in southwestern Ontario compared solid culture and direct and nested PCR (using *IS900* gene target) in milk with solid fecal culture, and detected more positive samples than did the current study. However, the Se results for milk, from the study by Gao et al. (2009), may have been overestimated by using a reference standard of solid fecal culture, which is known to have low Se. From cows previously test-positive on fecal culture, milk enzyme linked immunosorbent assay (**ELISA**), or serum ELISA, they reported a Se of 41.8% with fecal culture, 34.6% with milk culture, 28.4% with milk PCR, and 53.7% with milk nested PCR. In contrast, a study by Slana et al. (2008), found a Se of 35% with solid culture of milk and a maximum Se of 77.8% with milk qPCR. A recent study of Iranian farms found qPCR (using the *F57* gene) to be 10 times more sensitive than culture (using Middlebrook 7H11 media) in detecting MAP in milk samples (Hanifian et al., 2013). Furthermore, previous studies have also found that infected cows may shed between 2 to 9 CFU MAP per 50 ml milk (Sweeney et al., 1992; Rademaker et al., 2007), and that affected clinical cows shed up to 100 CFU per ml of milk (Giese and

Ahrens, 2000). Our Se estimates were within the range of other studies, possibly resulting from our choice of target condition and reference standard.

Bradner et al. (2013b) stress that more MAP would be shed into milk and colostrum as the disease progresses in the infected cow. Because MAP is an intracellular bacterium, able to reside within macrophages and resist phagocytosis, a higher burden in colostrum may be possible (Streeter et al., 1995). In our study, only four of the 36 MAP-infectious cows identified for colostrum sampling were culture-positive in colostrum, with three detected by broth culture and one by solid culture. Therefore, our improved Se with qPCR (approximately 35%) in colostrum was promising, as Pithua et al. (2011b) found that not using colostrum from infectious cows could lead to 18.2% lower risk of calves being exposed to MAP through colostrum. Additional research with larger sample sizes and, perhaps, comparisons of various techniques for detection of MAP in colostrum is recommended.

The selection of our case definition was based only on cows that were shedding MAP in their feces at least once within a one year period prior to the start of this study. Using this criterion to label cows as MAP-infectious, a potential bias may have been introduced in our definition for the Se of the three assays. Possibly, some of these cows were labelled as infectious when they may have been only intermittent or passive shedders (Nielsen and Toft, 2008). As the majority of cows were shedding detectable amounts of MAP during the study period, our case and target conditions, using fecal pathogen detection methods, were reflective of MAP-infectious cows following the disease stage criteria of Nielsen and Toft (2008) and Gardner et al. (2011).

Furthermore, there are no standard shedding categories for milk and colostrum in the literature. It is possible that, due to the difficulty in culturing MAP in these samples as compared to fecal samples, as well as the inherent lower bacterial concentration in these samples, the shedding categories we chose are over-reaching. However, we used the same shedding category cut-points for milk and colostrum samples as for fecal samples in order to compare CFU and days-to positive counts with some measure of similarity.

3.5.2 Concurrent Milk and Fecal Detection Patterns

Overall, our study found that when milk and feces were sampled concurrently, approximately one-third of MAP detected in feces was simultaneously detected in milk, regardless of the assay used (Table 3.3). Again, literature results vary regarding milk and fecal agreement (Nielsen and Toft, 2008; Pinedo et al., 2008; Gao et al., 2009), as techniques and methods differ between studies. A recent study, by Khol et al. (2013), in a low prevalence herd (9.0%, as previously determined by fecal qPCR testing) employed ELISA testing of blood samples, qPCR testing of fecal samples, as well as ELISA and qPCR testing of individual and bulk tank milk samples. They reported a significant correlation between MAP shedding in milk and feces, yet warned that shedding into milk may only occur for a limited time and may often be too low for detection even with PCR. It has been suggested that an increase in detectable shedding of the bacterium into milk should correspond with the level of fecal shedding and, ultimately, the stage of disease (Sweeney et al., 1992; Streeter et al., 1995). In our data, few cows had a high concentration of detectable MAP in their milk. Gao et al. (2009) suggested that, because

shedding in milk and feces may not consistently coincide, both milk and fecal culture should be used concurrently to avoid incorrectly identifying low-shedding cows as MAP test-negative. In our data, approximately 10% of milk broth cultures detecting low MAP shedding was observed when concurrent fecal broth cultures were negative. Therefore, colostrum, milk, or waste milk from high-risk herds or known MAP-positive herds could be a means of MAP transmission to calves even when concurrent fecal testing of those cows is negative. In these cases, it may be warranted to include milk testing (particularly with qPCR) as part of the herd's control program.

3.5.3 Seasonal and Lactational Patterns in Detection Ability

In our study of MAP-infectious cows, we did not find any significant associations between lactation stage and detection with any of the assays for milk samples. However, Bradner et al. (2013b) reported increased MAP detection in milk at the beginning of lactation (0 to 60 DIM) in affected Johne's diseased cows, and Stabel et al. (2014) reported the same for subclinical and affected cows. For milk samples, our raw data showed higher qPCR sensitivity over both culture methods across the lactation stages. This difference was more pronounced after 100 DIM and particularly after 240 DIM. Because of the long data collection period, DIM may have reflected progression of the disease within our study population of infectious cows.

Our study noted that the greatest disagreement between simultaneous shedding in feces and in milk was seen in the fall months (Table 3.3). In contrast, the best agreement was seen in the summer months, when there was highest qPCR detection ability in milk but lower qPCR detection ability in feces (Chapter 2). Our results of a higher detection

of MAP in raw milk samples during summer (July through September), in Atlantic Canada (Figure 3.1), contradict the study from England and Wales by Millar et al. (1996) which found that seasonal patterns in MAP detection in retail milk occurred in the peak periods from September to November and January to March for their regions. Although seasonal patterns would depend on geographical location, results can be extrapolated to various locations with areas of similar weather patterns. Crossley et al. (2005) also stress that milk policy and financial reasons involving milk supply and demand can determine whether MAP-infectious cows are kept on farm during certain months rather than being culled, and this could bias seasonal results. Furthermore, increased stress levels due to crowding, increased milk production, calving, changes in feeding practices and herd management, changes in environment (pasture), adverse weather conditions, and body condition may also lead to seasonal trends (Jørgensen, 1977; McKenna et al., 2004; Crossley et al., 2005). In addition to seasonal trends for MAP detection with qPCR in milk samples, our study also noted a trend for higher solid culture contamination in summer. Literature suggests that culture decontamination failures may be due to diet and farm location and, therefore, likely to be affected by clustering (Whitlock et al., 1989; Whittington, 2009). Little is still known about such possible causal factors for shedding and decontamination failure patterns, particularly for milk. However, this knowledge is important to enhance specific herd Johne's disease control management and diagnostic protocols, and warrants further research. In our study, although the number of case cows selected per farm varied, the low number of farms recruited led us to including farm as a fixed predictor in the model to account for possible confounding, but the inclusion of farm did not affect the point estimates of the

predictor for season. Our study supports the use of milk and colostrum testing, particularly with qPCR methods, in seasons where a higher likelihood for individual and environmental stress may occur, as well as seasons where milk concentration is decreased.

3.5.4 Conclusion

As calves have the highest risk of infection, one of the key management strategies in a Johne's control program is reduction of the spread of MAP by feces, milk, and colostrum. In this study, we analyzed milk and colostrum samples collected over a 12 month period from a group of previously identified MAP-infectious dairy cows. Traditional culture methods can take from less than two months up to four months, depending on culture method, to obtain a result, which can be futile for decreasing calf exposure in the calving pen. In addition, results from samples collected several months earlier to calving are not necessarily indicative of those cows being infectious at calving, and negative fecal tests did not imply negative milk or colostrum assays and vice versa. Therefore, our data indicated that qPCR, which can provide a result within 24 hours if required, showed an improved sensitivity as compared to culture methods. Furthermore, qPCR could be used to test both fecal samples of dairy cows in the dry season prior to calving, to evaluate potential risk of MAP-shedding in feces, and to test colostrum and early lactation milk prior to use or prior to storage, to evaluate risk of MAP exposure to calves via these routes.

Our study also highlighted a seasonal pattern for increased qPCR detection in milk samples collected during summer months, as well as more synchronization between

milk and fecal shedding in summer. Understanding patterns of detectable MAP shedding in milk and colostrum can reveal more efficient detection strategies for these samples. Future research should include repeating seasonal assessments on MAP detection ability over a several-year period, while monitoring other management and cow-level predictors, and using larger sample sizes, particularly for colostrum analyses.

3.6 References

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Table 3.1. Sensitivity (%) of solid culture (Herrold's egg yolk media with mycobactin J), broth culture (TREK ESP system; Thermo Scientific, Ohio), and real-time polymerase chain reaction (qPCR; VetAlert; Tetracore, Maryland) for fecal, milk, and colostrum samples collected monthly for a period of 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

	Solid culture	Broth culture	qPCR
Feces	46.7 (40.7-52.7[a]) [270[b]]	55.0 (49.3-60.7) [298]	77.4 (72.7-82.2) [297]
Milk	13.4 (9.5-17.3) [291]	14.1 (9.9-18.4) [262]	21.5 (16.8-26.2) [293]
Colostrum	3.0 [33]	10.7 [28]	35.3 [34]

[a]95% confidence interval, listed if more than 40 observations available.
[b]Number of observations.

Table 3.2. Percentage of solid culture (Herrold's egg yolk media with mycobactin J) and broth culture (TREK ESP system; Thermo Scientific, Ohio) outcomes across shedding levels for fecal, milk, and colostrum samples collected monthly for a period of 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

		Negative	Low shedding	Moderate shedding	High shedding
Solid culture[a]	Feces	53.3 (45.3-59.3[c]) [144[d]]	15.9 (11.5-20.3) [43]	8.1 [22]	22.6 (17.6-27.6) [61]
	Milk	86.6 (82.7-90.5) [252]	9.3 [27]	0.7 [2]	3.4 [10]
	Colostrum	97.0 [32]	0	0	3.0 [1]
Broth culture[b]	Feces	45.0 (39.3-50.6) [134]	30.9 (25.6-36.1) [92]	9.1 [27]	15.1 (11.0-19.2) [45]
	Milk	85.9 (81.6-90.1) [225]	11.8 [31]	2.3 [6]	0
	Colostrum	89.3 [25]	10.7 [3]	0	0

[a]Solid culture: low (< 10 CFU per culture tube), moderate (10 to 50 CFU), and high (> 50 CFU) (Crossley et al., 2005).
[b]Broth culture: low (> 28 days to a positive signal), moderate (21 to 28 days), high (< 21 days) (Shin et al., 2000; Shin et al., 2001).
[c]95% confidence interval, listed if more than 40 observations available.
[d]Number of observations at the corresponding shedding level.

Table 3.3. Percentage of positive milk sample results obtained from solid culture (Herrold's egg yolk media with mycobactin J), broth culture (TREK ESP system; Thermo Scientific, Ohio), and real-time polymerase chain reaction (qPCR; VetAlert; Tetracore, Maryland) when there was a concurrently positive fecal result, detected with one of the same three assay types, for samples collected over 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

	All seasons	Summer	Fall	Winter	Spring
Any milk test-positive[a]	36.5 (30.4-42.5[b]) [244[c]]	49.1 (35.8-62.4) [57]	28.9 (18.5-39.3) [76]	34.5 (21.9-47.0) [58]	35.8 (22.6-49.1) [53]
Milk qPCR+	22.7 (17.3-28.0) [238]	48.2 (34.8-61.6) [56]	8.4 (1.8-15.1) [71]	24.1 (12.8-35.4) [58]	13.2 (3.8-22.6) [53]

[a]Milk result classified as positive if there was a positive result on broth culture, solid culture, or qPCR assay.
[b]95% confidence interval.
[c]Total number of positive fecal results; fecal result classified as positive if there was a positive result on any one or more of broth culture, solid culture, or qPCR categories.

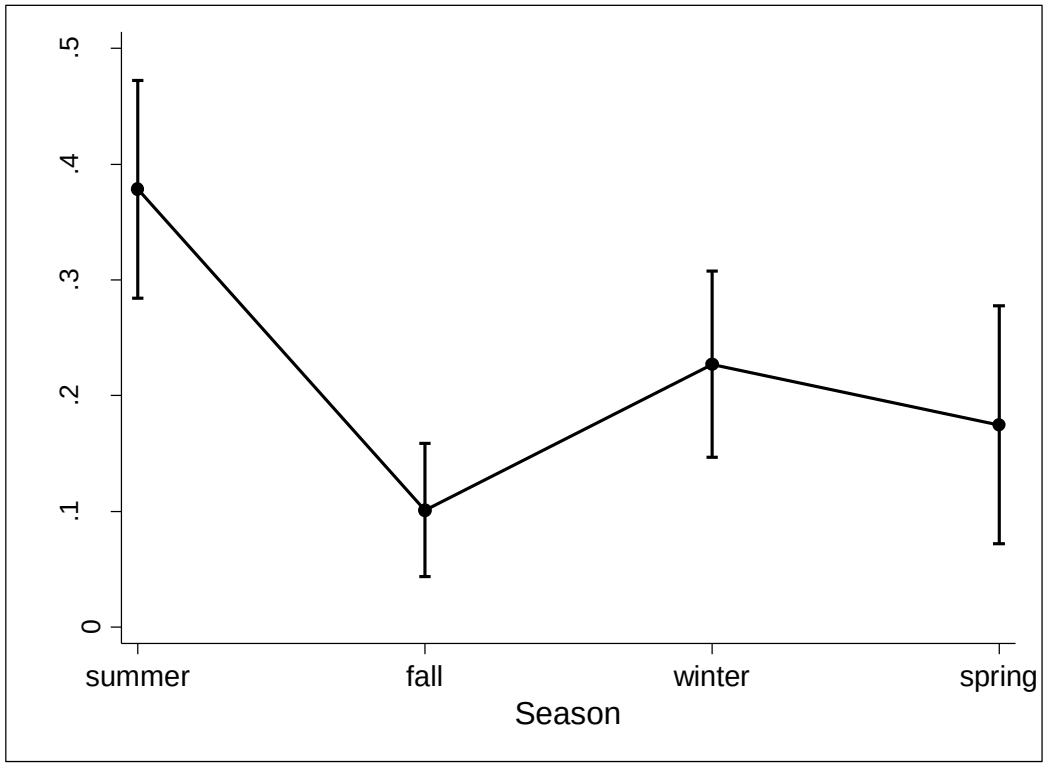

Figure 3.1. Predicted probability (%) with 95% confidence intervals for positive (+) direct real-time PCR (qPCR; VetAlert; Tetracore, Maryland) across seasons, for milk samples collected monthly over a period of 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

CHAPTER 4

THE ASSOCIATION OF FECAL SHEDDING, LACTATION STAGE, AGE, AND SEASON ON THE DETECTION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* INFECTIOUS DAIRY COWS IN ATLANTIC CANADA USING A COMMERCIAL MILK ELISA

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4.1 Abstract

Milk enzyme linked immunosorbent assays (ELISA) for Johne's disease in dairy cows are commonly used due to their low cost and quick processing for large numbers of samples. However, low sensitivity and imperfect specificity of the assay can impede detection of early disease stages. The objectives of this study were: (1) to compare the sensitivity and specificity of a commercial milk ELISA with solid and broth fecal culture and real-time polymerase chain reaction (qPCR) assays; and (2) to assess how detection of antibody concentrations in milk varies with changes in fecal shedding of the causative bacterium *Mycobacterium avium* subspecies *paratuberculosis*, host age, days in milk, and season. Monthly milk and fecal samples were collected over one year from 46 previously Johne's infectious (shedding) cows and 52 previously test-negative cows. Sensitivity of milk ELISA was 29.9% (95% CI: 24.8% to 35.1%), compared to 46.7% (40.7% to 52.7%) for fecal solid culture, 55.0% (49.3% to 60.7%) for fecal broth culture, and 78.4% (73.3% to 83.1%) for fecal direct qPCR. Specificity of the milk ELISA was 99.3% (97.5% to 99.9%). The milk ELISA sensitivity increased as fecal shedding increased and as host age increased. Furthermore, milk ELISA results both improved with increasing number of days in milk and were significantly higher in winter (January through March) than in summer (July through September). Improving the knowledge of factors affecting the interpretation of ELISA results could benefit Johne's disease management programs.

4.2 Introduction

Johne's disease, or paratuberculosis, has spread worldwide and is now endemic in Europe and North America (Barkema et al., 2010). Considering this disease can be transmitted silently and is often undiagnosed, paratuberculosis can have long-term devastating effects within a herd. As a production-limiting disease of dairy cattle, Johne's disease can have a substantial financial impact for dairy farmers (Tiwari et al., 2006). Caused by the organism *Mycobacterium avium* subspecies *paratuberculosis* (**MAP**), Johne's disease manifests clinically as chronic enteritis, including signs of diarrhea, weight loss despite a normal appetite, and decreased milk production (Fecteau and Whitlock, 2010). Paratuberculosis has a long incubation period, ranging from two to ten years, in which silent bacterial transmission can occur (Fecteau and Whitlock, 2010).

Nielsen and Toft (2008) described three general rankings for MAP-positive cows: MAP-infected cows that carry the organism; MAP-infectious cows that shed detectable amounts of the organism; and MAP-affected cows that present with clinical signs of the disease. MAP-infected cows can progress into MAP-infectious stages, often initially with intermittent bacterial shedding in feces, before becoming clinically MAP-affected with increased bacterial shedding (2008). Some cows in the MAP-infected stage can have an antibody response (prior to fecal shedding and clinical signs) that can be detected via an enzyme linked immunosorbent assay (**ELISA**). Although MAP-infectious cows may also have no detectable antibodies, ELISA sensitivity (**Se**) generally improves with increased bacterial shedding and clinical stages of disease

(Carpenter et al., 2004; Nielsen and Toft, 2006; Tiwari et al., 2006). The presence of antibodies can therefore be predictive of higher risk for MAP fecal shedding (Nielsen, 2008; Lavers et al., 2014). However, determinants of when antibodies to MAP become detectable in an infected cow are not well understood. In one study, a humoral response could be detected an average of one year prior to confirmed fecal shedding (Nielsen, 2008), but this may be highly variable, and ELISA is known to have imperfect specificity for MAP infection (Nielsen and Toft, 2008). Consequently, ELISA results (milk or serum) should be evaluated carefully, and it is often recommended to confirm results with direct detection methods (Benedictus et al., 1987; Nielsen et al., 2002c; Nielsen, 2010).

There are two broad categories of diagnostic tests available for Johne's disease detection and monitoring: tests that detect the bacterium (culture) or the bacterial DNA (polymerase chain reaction), and tests that detect the host's immune response to the bacterium (ELISA) (Tiwari et al., 2006). Culture is often considered the reference standard detection method, particularly fecal culture, as tissue culture is more invasive and impractical for most research studies (Tiwari et al., 2006; Bölske and Herthnek, 2010). However, cultures are dependent upon the characteristically slow growth for MAP, with incubation times ranging from seven weeks with liquid cultures to 16 weeks with solid cultures (Bölske and Herthnek, 2010).

Antibody ELISA is a much more time-efficient, cost-effective assay than fecal culture methods. However, Se of ELISA is generally poor (29% to 61%), with imperfect specificity (**Sp**) from 83% to 100% (Nielsen and Toft, 2008). McKenna et al. (2005) reported Se <10% for absorbed serum ELISAs, with tissue culture as a reference

standard. Depending on the reference standard used (tissue culture, previous fecal culture, concurrent fecal culture, clinical disease), prevalence of MAP in a herd, and ELISA kit and methodology, Se can vary greatly (Lombard et al., 2006). Nielsen and Toft (2006) also stressed the importance of relating the Se and Sp to the purpose of testing, and knowing whether infected, infectious, or affected cows are targeted for diagnosis. These targets correspond to the natural progression of the disease, and the inherent Se of all diagnostic modalities tends to improve as animals progress along the disease pathway.

Generally, improved ELISA Se should occur with increasing age or increasing parity, as this should inherently coincide with increased MAP shedding and clinical signs (Toft et al., 2005; Nielsen et al., 2013). Nielsen et al. (2002a) found an increase in Se for milk ELISA at the beginning of lactation, as compared to an increase in Se for serum ELISA at the end of lactation. The increase in milk ELISA Se in very early lactation could be related to the presence of high amounts of colostral antibodies (Nielsen et al., 2002b; Nielsen and Toft, 2012). For MAP-infected cows, the presence of cell-mediated immunity may result in poor detection of a humoral result, as one study found low humoral antibodies detected at 60 days in milk (**DIM**) for MAP-infected cows (Nielsen et al., 2002a). Therefore, there is need to carefully assess how ELISA Se varies across lactation stages in light of Johne's disease stage, the presence of nonspecific colostral antibodies, and milk dilution effects (Nielsen and Toft, 2012). A recent study by Cazer et al. (2013) assessed seasonal effects on bulk tank milk ELISA results and detected increased MAP antibodies in bulk tank milk during summer and a decrease during winter. This effect may be dependent upon seasonal calvings or a

humoral immunity peak in response to increased exposure to MAP during specific seasons (Collins et al., 2005).

As management is the key to controlling Johne's disease on a farm, adequate diagnostics are essential (Garry, 2011). Due to the inadequacies of current diagnostics and the intermittent bacterial shedding in subclinical Johne's disease, the identification of any antibody detection pattern related to host-factors (age, parity, lactation stage) or environmental-factors (season) can be beneficial for the dairy industry when using ELISA assays. The need for continued research regarding Johne's disease and finding more efficient diagnostic strategies for the dairy industry is fueled by the production-limiting effects of this disease, as well as its status as an animal health priority and its potential zoonotic link to human Crohn's disease (Barkema et al., 2010).

Therefore, the objectives of this study were to compare Se of milk ELISA with solid and broth fecal culture and real-time polymerase chain reaction (**qPCR**) assays for MAP-infectious cows, and to assess how detection of antibody concentrations in milk varies with changes in fecal shedding of MAP, host age or parity, DIM, and time of year (season).

4.3 Materials and Methods

4.3.1 Farm and Cow Selection

MAP-positive Holstein dairy herds in New Brunswick (four) and Prince Edward Island (three), Canada, were recruited for this study, with within-herd MAP prevalence for these farms (83 to 490 cows per herd) ranging from 3% to 15% in the previous year

(C. Lavers; Atlantic Veterinary College (**AVC**), Charlottetown, PE, Canada, personal communication). For this study, the target condition was MAP-infectious as defined by fecal MAP shedding detected by culture methods (Nielsen and Toft, 2008). The case definition was any cow that was MAP-infectious at least once during a one year period prior to the start of this study, as determined from a companion study (Lavers et al., 2013) in which MAP-positive cows were identified using pooled fecal broth culture confirmed with acid fast stain and qPCR. Any positive pools had individual fecal samples further cultured. The term MAP-infectious will continue to be used in the remainder of the text to describe these cows. A total of 46 MAP-infectious cows were identified for milk and fecal sampling for this study.

To examine the milk ELISA Sp for Johne's disease with the selected commercial ELISA for this study, a control group of cows from the same farms was identified for milk sampling. No feces were sampled for this group as the Sp for both fecal culture and qPCR was assumed to be 100%. The target condition for this group of 52 cows was a MAP test-negative herd-mate. The control group case definition was a herd-mate, matched as closely as possible by age, parity, DIM, and reproductive status to a MAP-infectious cow. These cows were previously confirmed (Lavers et al., 2013) as repeatedly test-negative through fecal broth culture (confirmed with acid fast stain and qPCR), milk ELISA, and serum ELISA. If any of these cows showed a positive milk ELISA score during the study period, fecal broth culture was performed for three consecutive months. If the cow was subsequently confirmed as MAP-infectious by positive fecal culture results, then a new control cow was selected to replace it.

4.3.2 Sample Collection

The Animal Care Committee (University of Prince Edward Island, Charlottetown, PE, Canada) approved all animal protocols prior to the start of this study. Milk and fecal samples were collected monthly, during lactation periods, for up to 12 months or as long as the cows remained in the herds, from July 2010 to December 2011. Samples of feces were collected by the project personnel via rectal palpation, using individual, non-lubricated rectal sleeves. Clean milk samples were collected either by the project personnel or by the farmers. Until processing, all samples were frozen at -80°C in the USDA proficiency tested Maritime Quality Milk Laboratory (AVC, University of Prince Edward Island, Charlottetown, PE, Canada).

4.3.3 Laboratory Procedures

4.3.3.1 Fecal Solid Culture

Herrold's egg-yolk medium (**HEYM**) slants (Becton, Dickinson, and Company, Sparks, Maryland), supplemented with mycobactin J, amphotericin B, naladixic acid, and vancomycin, were used, following methods described by Stabel (1997) with modifications. In short, 30 mL of half-strength Brain Heart Infusion (**BHI**) with 0.9% hexadecylpyridinium chloride monohydrate (Sigma Chemical Company, St. Louis, Missouri) (**HPC**) was mixed with 3 g of feces and allowed to settle for 30 minutes at room temperature (21˚C). Subsequently, 15 ml of the supernatant (placed in a new 50 ml polypropylene tube) was centrifuged at 1,700 x *g* for 20 minutes at room temperature. After discarding the supernatant, the pellet was re-suspended with 30 mL of the 0.9% HPC-BHI solution and incubated overnight at 37˚C. After being centrifuged at 1,700 x *g*

for 20 minutes at room temperature and discarding the supernatant, the pellet was re-suspended with 100 µL of 0.9% saline solution. The media was then inoculated with 100 µL of the re-suspended sample, and the slant was placed at a slight incline at 37˚C. When the liquid dried, the cap was tightened, and the slant was placed vertically in a rack at 37˚C for 84 days. Weekly examinations using a dissecting microscope were performed, and the colony forming unit (**CFU**) recorded until >100 CFU per tube were observed, or until growth of competitive bacteria and fungi covered any detectable MAP colonies.

4.3.3.2 Fecal Broth Culture

With the TREK system (TREK ESP® culture system II, Thermo Scientific, Oakwood Village, Ohio), MAP growth is recorded as a days-to-positive reading signaled by decreased pressure detected in the media bottle. In short, 2 g of thawed fecal sample was mixed with 35 mL of sterile water for 10 minutes on an automatic shaker (Mistrel Multi Mixer 4600, Barnstead Lab-Line, Melrose Park, Illinois) and incubated for 30 minutes at room temperature. Five mL from the top one-third of the solution was mixed with 25 mL of 0.9%HPC-BHI solution and incubated overnight at 37˚C. After centrifugation at 1,500 x *g* for 20 minutes, the pellet was re-suspended with 1 mL of an antibiotic brew (18.5 mg/mL BHI, 975 µl/mL deionized water, 100 µg/mL vancomycin, 100 µg/mL nalidixic acid, and 50 µg/mL amphotericin B) and incubated again overnight at 37˚C. Then in the mycobottle (Para-JEM® broth bottle, Thermo Scientific, Nepean, Ontario, Canada), 1 mL of the decontaminated sample was mixed with 2.5 mL of a brew of 1 mL Growth Supplement (TREK ESP® culture system II, Thermo Scientific,

Oakwood Village, Ohio), 1mL Egg Yolk Supplement (TREK ESP® culture system II, Thermo Scientific, Oakwood Village, Ohio), and 1 mL Antibiotic Supplement (TREK ESP® culture system II, Thermo Scientific, Oakwood Village, Ohio). The bottle was then placed in the TREK incubator until a positive signal was reached, up to a maximum of 49 days.

4.3.3.3 Fecal Direct qPCR

Procedures followed the recommendations of the Tetracore kit (VetAlert™ Johne's Real-Time PCR kit, Tetracore®, Rockville, Maryland) targeting the *hspX* gene. Two g of fecal sample was mixed with 35 mL of sterile distilled water, placed on an automatic shaker for 15 minutes, and incubated for 30 minutes at room temperature. Then the top 20 mL of the solution was centrifuged at 2,500 x *g* for 10 minutes at room temperature, and the pellet was re-suspended with 1 mL of 1xTE buffer (10 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA). For DNA extraction, 1 mL of the solution was bead-beat (Mini-Beater 8, BioSpec Products, Bartlesville, Oklahoma) in a cell-disruption tube containing sterile glass, for 5 minutes at 4,800 oscillations per minute, then centrifuged for 10 minutes at 16,000 x *g.* The supernatant was mixed with 100 µL Nucleic Acid (NAB™) Buffer and centrifuged at 1,200 x *g* for 3 minutes. The pellet was re-suspended with 560 µL of Binding Buffer, incubated for 10 minutes at room temperature, and mixed with 560 µL of 100% ethanol. Then, 630 µL of the sample at a time was placed in a spin column and centrifuged at 5,200 x *g* for 1 minute, followed by adding 500 µL of Wash Buffer A (centrifuged at 5,200 x *g* for 1 minute), 500 µL of Wash Buffer B (centrifuged at 12,000 x *g* for 3 minutes), and no buffers (centrifuged at 16,000 x *g* for 1

minute). Then 50 µL of deionized water was added, incubated for 1 minute at room temperature, and centrifuged at 5,200 x *g* for 1 minute to elute the DNA. For the fluorogenic probe hydrolysis assay, 22.5 µL Master-mix was mixed with 2.5 µL of the eluted DNA in a thermocycler reaction tube. Positive and negative control samples were also included in each qPCR run in the thermocycler (Cepheid SmartCycler™ II Thermocycler, Cepheid, Sunnyvale, California). An enzyme activation step (95˚C) was followed by a two-step cycling reaction (95˚C and 62˚C). Positive control cut-off was set between 20 to 26 threshold cycles (**Ct**). Positive samples were <42 Ct.

4.3.3.4 Culture Confirmation

Acid fast stain was performed for all broth and solid cultures to confirm for positive MAP. Any positive culture or AFS positive samples were further confirmed with qPCR. For broth cultures, the bottle was placed in an automatic shaker for 5 minutes and 1 mL of the culture was added to a cell-disruption tube. For solid cultures, two or three colonies were collected from the media with a sterile loop, mixed with 1 mL of sterile 0.9% saline in a sterile centrifuge tube, with 1 mL of the re-suspended colonies subsequently placed in a cell-disruption tube. The methods described above for DNA elution and qPCR were then followed.

4.3.3.5 Milk ELISA

Procedures followed the recommendations of the Paracheck kit (PARACHEK® 2 ELISA kit, Prionics AG, Schlieren-Zürich, Switzerland). This indirect ELISA includes an absorption phase with *Mycobacterium phlei* to eliminate any cross-reacting

antibodies to other mycobacteria that could result in false positives (Yokomizo et al., 1985). As the kinetic detection method was used, the positive cut-off value was determined as the average value of the two negative control OD values plus 0.100. The adjusted score results (sample raw OD minus average of the two negative control OD values) were set at <0.07 as negative, 0.07 to 0.1 as suspicious, and >0.1 as positive. Any results within the suspicious category were re-run to classify as either positive or negative. If they stayed in the 0.07 to 0.1 range, they were classified as negative as per kit instructions.

In each of the first two wells of a 1.2 mL dilution plate, 10 μL of negative control was added, followed by 10 μL of positive control in the next two wells, followed by 150 μL of milk samples per each subsequent well. Then 190 μL of sample diluent (including *Mycobacterium phlei*) was mixed into each of the controls, and 150 μL of sample diluent was mixed with each sample. The plate was incubated at room temperature for 30 minutes. From each well, 100 μL was transferred to the MAP antigen-coated ELISA microtitre plate. The covered plate was incubated at room temperature for 45 minutes to allow MAP-specific antibodies to bind to the MAP antigens coated on the plate, then washed six times with wash buffer (20x concentrate mixed with 19 parts distilled water) in the washer (BioTek® ELx 405 ELISA Washer, Thermo Fisher Scientific, Waltham, Maine) to remove unbound proteins. Then 100 μL of conjugate (a secondary antibody conjugated to an enzyme that can produce a color signal), diluted 1:100 with conjugate diluent, was added to each well, followed by the covered plate being incubated at room temperature for 30 minutes, then washed again as above. Afterwards, 100 μL of enzyme substrate solution (in order for the enzyme to release the color signal) was added to each

well, and the plate placed in the plate reader (BioTek® Power Wave XS ELISA Reader, Thermo Fisher Scientific, Waltham, Maine). The positive control absorbance was read at 630 nm until it reached between 0.35 to 0.40 OD, using the ELISA analysis software (Gen5™ Data Analysis Software, BioTek, Winooski, Vermont). Then 50 μL stop solution was added to each well, and the absorbance of each well read at 450nm. For the results to be valid, negative controls had to be <0.200 OD and not vary by more than 0.04 OD. The positive controls had to be between 0.900 to 1.200 OD and not deviate by more than 30%.

4.3.4 Statistical Analysis

Statistical analyses were done using STATA (STATA®/IC Version 12, StataCorp LP, College Station, Texas), Minitab (Minitab® 16.2.2, Minitab Inc., State College, Pennsylvania), and MLwiN (MLwiN®, Centre for Multilevel Modelling, Bristol, United Kingdom) software using a *P*-value <0.05 as a cut-off for statistical significance. Sensitivity (proportion of observations that were positive for the previously determined MAP-infectious cows) and Sp (proportion of observations that were negative for the previously determined MAP test-negative herd-mates) for ELISA results were recorded along with a 95% confidence interval (**CI**). Analysis was limited to data from cows with <400 DIM to stay within a typical lactation length and because data were sparse above this threshold. ELISA Se proportions were compared to Se of three fecal assays (solid and broth cultures and qPCR). Additionally, agreement analyses, using the Cohen's Kappa and McNemar's exact test (Dohoo et al., 2009), were calculated between dichotomous milk ELISA and fecal results. Continuous ELISA scores were also

compared to fecal shedding levels. For solid culture, low shedding related to <10 CFU per culture slant, moderate shedding from 10 to 50 CFU per slant, and high shedding >50 CFU per slant (Crossley et al., 2005). For broth culture, high shedding related to <20 days to positive, moderate shedding between 21 and 28 days, and low shedding between 29 and 49 days (Shin et al., 2000, 2001). For qPCR, high shedding related to <26 Ct, moderate shedding between 26 to 30 Ct, and low shedding between 30 to 42 Ct, according to previous work (see Chapter 2).

For milk samples, Se and score values were analyzed over age, parity, lactation stage, and season. A two-level (cow and farm) hierarchical mixed linear regression model for continuous ELISA scores was used for the analysis, using an autoregressive (AR1) residual correlation structure for the relationship between times within tests within cows. Univariate analysis was explored first, followed by the multivariable model, including any pertinent interaction terms. Analyzed predictors of interest were season (categorical), DIM (continuous), age (categorical), and parity (categorical). The collinear age and parity variables were analyzed in separate models. Parity was grouped into first, second, third, fourth, and >fifth parity. Age was categorized into ages two and three years, four years, five years, six years, and greater than seven years. Finally, season was categorized into July through September for summer, October through December for fall, January through March for winter, and April through June for spring. Contrasts were analyzed for any significant predictors and reported with Bonferroni-adjusted *P*-values for multiple comparisons.

4.4 Results

4.4.1 Descriptive Data

For both infectious and test-negative groups, cows ranged in ages from two to nine years (median = four years) and from first to seventh parity (median = second parity). From the 46 MAP-infectious cows (range of one to eleven observations per cow over the study period), there were total observations of 304 milk ELISA, 270 fecal solid culture, 298 fecal broth culture, and 297 fecal qPCR. From the control group, there were 327 milk ELISA observations. Table 4.1 shows the consistency of each methodology by the number of MAP-infectious cows in positive-result percentiles across each of the four testing methods. Of the 46 cows that were previously found to be infectious, only one cow had no shedding detected with a fecal pathogen detection method during the study period. This cow was only tested once and then culled. Of the remaining 45 cows, 14 cows had detectable fecal MAP shedding from 25.0% to 87.8% of their sampling times. Kappa between milk ELISA and fecal culture techniques indicated moderate agreement, with 0.57 for solid culture ($P<0.01$) and 0.46 for broth culture ($P<0.01$); but there was poor agreement (0.19, $P<0.01$) with fecal direct qPCR. McNemar's test was highly significant ($P<0.01$) for all of these assay comparisons for milk and fecal samples.

Monthly variation in the detection ability of the ELISA for milk samples and of the three testing methods (solid culture, broth culture, and qPCR) for fecal samples from the MAP-infectious cows is depicted in Appendix A.

4.4.2 Detection Ability of Assays

Overall Se values for milk ELISA, as well as for the three fecal diagnostic methods are listed in Table 4.2. One control cow was identified as a MAP-infectious cow after two consecutive milk ELISA observations confirmed with fecal culture and was subsequently removed from evaluation of milk ELISA Sp. Specificity was calculated from the test-negative group as 99.3% (95% CI: 97.5% to 99.9%). There were three cows with false positive results (three observations).

4.4.3 Association with Fecal Shedding, Age, or Parity

A higher proportion of positive results for milk ELISA was seen when fecal shedding was greater (Table 4.3). This trend was significant for high shedding detected with fecal solid culture (high versus negative, $P < 0.01$; high versus low, $P<0.05$; high versus moderate, $P = 0.09$) and qPCR (high versus negative, $P<0.01$; high versus low, $P<0.01$; moderate versus negative, $P = 0.06$). There was also a trend toward improved Se and greater mean ELISA scores with increasing age (Table 4.4). However, after Bonferroni corrections for multiple comparisons, this trend was not significant. A similar trend was also seen with increasing parity (data not shown).

4.4.4 Seasonal and Lactational Patterns in Detection Ability

Because the ELISA scores ranged from -0.17 to 2.42 and were highly right skewed (mean 0.157, median -0.06), the outcome for the mixed linear model was best transformed to the inverse square root for the regression analysis in order to meet the assumptions of the linear model. As the predictors for age and parity were highly

correlated (r = 0.85, $P < 0.01$), both variables were not analyzed in the same model. In the mixed linear regression model, predictors of ELISA score included age ($P < 0.05$), season ($P < 0.05$), and DIM ($P < 0.01$). The model with parity was similar (data not shown). Predicted back-transformed marginal scores (Figure 4.1) improved toward end of lactation and were more likely to cross the positive threshold (0.1) with increasing age. Scores were significantly higher for winter ($P < 0.05$) than for summer. Numerically, the changes in scores were often quite small and did not necessarily result in an increase in scores above the positive threshold due to few observations in some categories among season, age, and lactation stages.

4.5 Discussion

4.5.1 Detection Ability of Milk ELISA

Milk ELISA for Johne's disease has poor cow-level predictive values due to low Se and imperfect Sp, when combined with low within-herd prevalence (Tiwari et al., 2006). The current analysis performed in lower MAP prevalence herds (3% to 15%) supports reports of lower milk ELISA Se than standard fecal diagnostics (Table 4.2), with a milk Se of approximately 30% and Sp of 99%. Similarly, a literature review by Nielsen and Toft (2008) assessed the six milk antibody ELISA studies done up to that time, and found that most of those studies targeted MAP-infectious cows with Se ranging from 29% to 61% and Sp from 83% to 100%. A study by Slana et al. (2008)

found high Sp along with Se ranging between 21% and 67%, using the same ELISA kit as the current study on individual cow milk samples. A literature review by Tiwari et al. (2006) reported milk Se between 51% to 84% and Sp between 92 to 96%. However, many of the reviewed studies used concurrent high fecal shedding (>10 CFU) as a reference standard as well as sampling from higher prevalence herds (>25% prevalence). McKenna et al. (2005) observed that serum ELISA Se was approximately two times greater if estimated against a reference standard of positive fecal culture than against tissue culture.

In the current Se study, only cows that were MAP-infectious the previous year were sampled, so the negative milk ELISA results were false negative under the case definition. It is possible that some of these cows may not have transitioned to a consistently detectable humoral immunity. Generally, ELISA Se should increase with each stage of disease and with increasing numbers of shed bacteria (Carpenter et al., 2004), which we observed in this study. There were two confirmed positive milk ELISA results, within the 327 observations from the control cows. These results came from the same cow in two consecutive months, even though fecal testing of this cow prior to the study did not detect MAP. Concerning the three confirmed false positive cows in this group, the possibility exists that they may not have been truly false, but may rather have been an early humoral response prior to detectable intermittent shedding (Nielsen, 2010). If these were true positives, then the Sp of the test approached 100%. Nielsen (2008) suggests that the best time to confirm the ELISA with fecal culture could be six to nine months post-ELISA, but cautions that cows with repeated positive results on ELISA testing are at higher risk of becoming MAP-infectious; therefore, the use of

repeated ELISA samples was recommended (Nielsen et al., 2002b). Furthermore, Nielsen (2008) hypothesized that the ability of cows to become high bacterial shedders could be predicted by the onset of detectable MAP antibodies.

Although it can be difficult to interpret Kappa in the presence of low prevalence (Byrt et al., 1993), this study found that there was only moderate agreement between milk ELISA and fecal culture tests, and slight agreement with the more sensitive fecal qPCR. These findings corresponded with the highly significant McNemar's tests, which indicated that the proportion of positive results between milk ELISA and any of the three fecal assays was significantly different. Hendrick et al. (2005) found that, despite a non-significant Se difference between an indirect milk ELISA and fecal culture, their level of agreement was greater than that between serum ELISA and fecal culture.

4.5.2 Association with Fecal Shedding, Age, or Parity

When individual shedding categories for fecal cultures were assessed (Table 4.3), there was a higher proportion of milk ELISA positive results and a 40% increase in the ELISA score for cows with concurrent heavy MAP shedding (>50 CFU on solid culture) than lighter shedding. Increased Se of ELISA with increased MAP shedding was also noted in a study of serum ELISA versus HEYM fecal culture and direct PCR (Clark et al., 2008). Although there are various studies done on comparisons between ELISA OD values and fecal shedding probabilities, the use of various study designs, target conditions, herd prevalence, and ELISA and culture methods makes comparison with these studies difficult (Lombard et al., 2006).

Increasing ELISA readings have been recorded over age and parity (Nielsen and Toft, 2002b; Toft et al., 2005). The current study did not detect any positive milk ELISA for two year old cows, and many scores were also negative in three year olds (Table 4.4). This pattern was a result of study design, as fecal culture positivity within the previous year was used for the case definition. A trend towards higher Se was seen in infected cows greater than five years old and especially older than seven years old (Table 4.4), indicating that the change to humoral immunity is more detectable in older cows. In a recent study using IDVET ELISA and looking at MAP-infected cows only, the Se over age also increased, but was generally higher than the Se reported in the current study and other studies (Nielsen and Toft, 2013). Several studies report higher Se after three years of age (Huda et al., 2004; Nielsen and Ersbøll, 2006), which is consistent with the current study results. One study found milk ELISA Se of 6% and Sp of 99.7% at age two, and a Se of 50% and Sp of 93% at age five in MAP-infected cows, but found no change between these ages for MAP-infectious cows (Nielsen and Toft, 2006). However, in the current study of MAP-infectious cows, just over 30% Se at age five was detected, and it did not exceed 50% until age seven. Nielsen and Toft (2006) also observed that the age of onset of shedding may be a better predictor of ELISA positivity than chronological age.

4.5.3 Seasonal and Lactational Patterns in Detection Ability

The present study detected improving milk ELISA scores in later lactation, and also found that the effect of lactation could not be separated from the effect of season, as the pattern predicted higher scores during winter months (Figure 4.1). However, the

variation in scores was sometimes numerically small and did not necessarily cross the positive threshold. In contrast, Nielsen et al. (2002a) found that milk ELISA Se increased at the beginning of lactation and serum ELISA at late lactation, but that study excluded cows greater than 290 DIM. An inherently higher presence of antibodies in colostrum could lead to higher antibody levels very early in lactation, even in non-infectious cows (Nielsen and Toft, 2002b). Therefore, milk antibody concentration may be greater both in early (first to second weeks) and late (greater than 45 weeks) lactation than in the third to twelfth weeks of lactation (Nielsen and Toft, 2002b; Lombard et al., 2006) due to colostral antibody levels, milk dilution effects, and Johne's disease stage (Nielsen and Toft, 2012).

4.5.4 Conclusion

Understanding patterns in MAP shedding and antibody concentration over time can lead to efficient testing of animals for enhanced control programs. Yet a lack of comparable scientific research on MAP shedding and immune patterns during lactation and over seasons in dairy cows could impede advances in the use of diagnostic tests in current MAP control programs within the dairy industry. The current study assessed seasonal and lactational patterns on MAP antibody concentrations in milk. However, our study was limited due to the choice of target and case conditions of MAP-infectious cows from the one year period prior to the present study. Milk ELISA results need to be cautiously interpreted relative to the target condition. Another limitation was the small sample size from low MAP prevalence farms, which may have resulted in low statistical power in some analyses.

Accurate knowledge of shedding and diagnostic patterns is vital for reducing MAP transmission risks and for development of improved diagnostic and screening protocols. The present data indicates overall milk ELISA Se of 30% and Sp of 99.3%, with increasing ELISA Se in relation to increased fecal shedding, increased age, and increased parity. Most notably, a significant effect for season (winter versus summer) and lactation stage (increasing DIM) on ELISA score values more clearly explains the variation over time and highlights areas that require further study in the understanding of this disease.

4.6 References

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Table 4.1. Number of cows in each positive-result category (percentiles) for fecal solid culture (Herrold's egg yolk media with mycobactin J), fecal broth culture (TREK ESP system; Thermo Scientific, Ohio), fecal real-time polymerase chain reaction (qPCR; VetAlert; Tetracore, Maryland), and milk ELISA (Paracheck; Prionics, Switzerland) for samples collected monthly over a 12 month period from a total of 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

Proportion of samples Positive (%)	Fecal Solid Culture	Fecal Broth Culture	Fecal qPCR	Milk ELISA
0	14	8	3	19
>0 to ≤25	6	7	1	6
>25 to ≤50	4	3	6	4
>50 to ≤75	3	3	7	3
>75 to ≤99	2	6	3	3
100	17	19	26	11
N[a]	46	46	46	46

[a]Total number of observations.

Table 4.2. Overall and shedding-level stratified sensitivity (%) of fecal solid culture (Herrold's egg yolk media with mycobactin J), fecal broth culture (TREK ESP system; Thermo Scientific, Ohio), fecal real-time polymerase chain reaction (qPCR; VetAlert; Tetracore, Maryland), and milk ELISA (Paracheck; Prionics, Switzerland) for concurrent samples collected monthly for 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

			Shedding levels		
	N[a]	Positive[b]	Low	Moderate	High
Milk ELISA	304	29.9 (24.8-35.1[c])			
Fecal solid culture[d]	270	46.7 (40.7-52.7)	15.9 (11.5-20.3)	8.1 (4.9-11.4)	22.6 (17.6-27.6)
Fecal broth culture[e]	298	55.0 (49.3-60.7)	30.9 (25.6-36.1)	9.1 (5.8-12.3)	15.1 (11.0-19.2)
Fecal direct qPCR[f]	297	77.4 (72.7-82.2)	58.2 (52.6-63.9)	10.1 (6.6-13.5)	9.1 (5.8-12.4)

[a]Total number of observations.
[b]Overall sensitivity regardless of shedding levels.
[c]95% confidence interval.
[d]Shedding categories determined with solid culture: low (<10 CFU per culture tube), moderate (10 to 50 CFU), high (>50 CFU) (Crossley et al., 2005).
[e]Shedding categories determined with broth culture: low (>28 days to a positive signal), moderate (21 to 28 days), high (<21 days) (Shin et al., 2000; Shin et al., 2001).
[f]Shedding categories determined with qPCR: low (30 to 42 Ct), moderate (26-30 Ct), high (<26 Ct).

Table 4.3. Proportion (%) of positive milk ELISA results (Parachek; Prionics, Switzerland) within each shedding category for fecal solid culture (Herrold's egg yolk media with mycobactin J), fecal broth culture (TREK ESP system; Thermo Scientific, Ohio), and fecal real-time polymerase chain reaction (qPCR; VetAlert; Tetracore, Maryland) for concurrent samples collected monthly over 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.[a]

	Shedding levels			
	Negative	Low	Moderate	High
Fecal solid culture[f]	6.2 (144[d]) [2.3-10.2[e]][a]	44.2 (43) [29.1-59.3][a]	45.4 (22) [24.1-66.8][a]	80.3 (61) [70.2-90.4][b]
Fecal broth culture[g]	3.7 (134) [0.0-7.0][a]	43.5 (92) [33.2-53.7][a]	51.8 (27) [32.6-71.1][a]	68.9 (45) [55.1-82.6][a]
Fecal direct qPCR[h]	4.7 (67) [0.0-9.5][a]	24.3 (173) [17.8-30.7][ab]	73.3 (30) [57.2-89.5][bc]	85.1 (27) [71.5-98.9][c]

[a-c]Significant differences between shedding levels per fecal assay (within same row) are represented by different superscript letters ($P < 0.05$).
[d]Total number of observations.
[e]95% confidence interval.
[f]Shedding categories determined with solid culture: low (<10 CFU per culture tube), moderate (10 to 50 CFU), high (>50 CFU) (Crossley et al., 2005).
[g]Shedding categories determined with broth culture: low (>28 days to a positive signal), moderate (21 to 28 days), high (<21 days) (Shin et al., 2000; Shin et al., 2001).
[h]Shedding categories determined with qPCR: low (30 to 42 Ct), moderate (26-30 Ct), high (<26 Ct).

Table 4.4. Sensitivity (%) and mean score results (%) of milk ELISA (Parachek; Prionics, Switzerland) within each age group for milk samples collected monthly over 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds.

Age (years)	N[a]	ELISA Sensitivity	Mean Score
2 and 3	90	25.6 (16.5-34.6[b])	0.09 (0.35[c])
4	78	20.5 (11.5-29.6)	0.08 (0.41)
5	51	33.3 (20.2-46.4)	0.23 (0.62)
6	55	32.7 (20.2-45.3)	0.22 (0.48)
>7	30	56.7 (38.6-74.8)	0.27 (0.50)

[a]Total number of observations.
[b]95% confidence interval.
[c]Standard deviation.

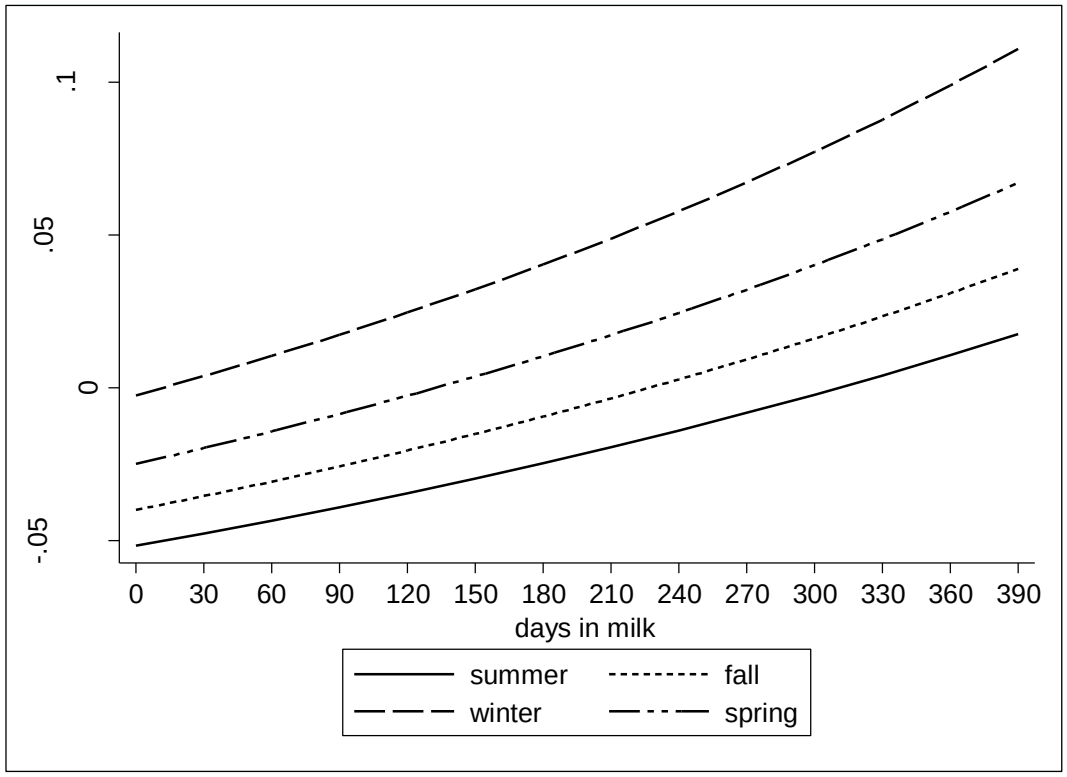

Figure 4.1. Predicted marginal adjusted scores for milk ELISA (Parachek; Prionics, Switzerland) across lactation months within each season for milk samples collected monthly over 12 months from 46 *Mycobacterium avium* subsp. *paratuberculosis* infectious cows from seven herds. Marginal scores were calculated on the mixed linear model with farm and cow level random effects and mean age (4.5 years).

CHAPTER 5

USE OF SPECIFIC *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* ANTIGENS FOR THE DEVELOPMENT OF EARLY DIAGNOSTIC TESTS FOR JOHNE'S DISEASE IN DAIRY COWS.

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5.1 Abstract

Paratuberculosis, or Johne's disease, is a chronic granulomatous enteritis of ruminants and a costly, production-limiting disease of dairy cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). To survive in the hostile environment within macrophages, MAP secretes a battery of proteins upon internalization to neutralize the immunological response of macrophages. One of the proteins important for the virulence of MAP is tyrosine phosphatase A (PtpA). MAP also possesses the protein tyrosine phosphatase B (PtpB), and the protein kinase G (PknG), both shown as ortholog virulent factors for the closely related bacterium, *Mycobacterium tuberculosis*. The PtpA inhibits phago-lysosome maturation and phagosome acidification in macrophages. Because colostrum is a macrophage-rich environment, it may be a risk of MAP infection for and transmission to calves and thus also a critical area to emphasize in farm control programs. Therefore, our objectives focused on developing cost-efficient, time-efficient, and effective early diagnostic tests based on these virulence proteins. This pilot effort looked first at modifying a novel enzyme linked immunosorbent assay (ELISA), particularly for colostrum, but also for milk and serum samples, and secondly modifying the cell-mediated interferon gamma (IFN-γ) assay for whole blood samples to identify the stimulation activity of MAP virulence proteins as specific antigens. Our efforts for developing the novel ELISA showed a response to PtpA especially in milk and to a cocktail of proteins, including PknG, for colostrum, but results were variable and not consistent. The production of IFN-γ was detected in IL-12 p40-potentiated PtpA and PknG whole blood samples.

However, due to inconsistent results in the adult infected and non-infected cows tested, coupled with a small sample size, results were not sufficient to determine the accurate effect of these virulence proteins. Nevertheless, this pilot study provides hopeful results, which are being further evaluated in ongoing work with both the novel ELISA and novel IFN-γ tests.

5.2 Introduction

The hallmark virulence activities of the bacteriological agent for paratuberculosis (Johne's disease), *Mycobacterium avium* subsp. *paratuberculosis* (**MAP**), are founded on its ability to thwart immunological attempts to clear the host of the infection and to coordinate the alterations of these complex immune pathways to gain intracellular survival (Bannantine and Stabel, 2002). The four foremost methods of mycobacterial survivability in macrophages are the inhibition of: (1) phagosome-lysosome fusion, (2) inhibition of phagosome acidification, (3) inhibition of antigen presentation, and (4) apoptosis (Koul et al., 2004). By altering normal macrophage activity, MAP can inhibit normal antigen processing and presentation and alter innate interferon gamma (**IFN-γ**) immune activity (Bach et al., 2011; Sweeney, 2011; Verschoor et al., 2010). There is not a wealth of information available for Johne's disease, regarding which proteins the bacterium secretes, so much information is translated from *Mycobacterium tuberculosis* research. The basic understanding is that a battery of proteins is secreted in order to orchestrate the immunological response elicited by macrophages upon infection, as well

as proteins necessary for the survival of the pathogen intracellularly, such as lipases that can be used by mycobacteria for basic metabolism and survival (Deb et al., 2006; Bach et al., 2011).

The establishment of a successful infection by MAP may rely on the secretion and action of specific proteins. Protein tyrosine phosphatase A (**PtpA**) of MAP, also known as low molecular weight MAP1985 (Li et al., 2005), has been reported to be actively secreted in macrophages within the first 24 hours post-infection (Bach et al., 2006) and actively involved in dephosphorylation of the host vacuolar sorting protein VPS33B, an essential protein required for phagosome-lysosome maturation (Bach et al., 2008). It has been suggested that PtpA is continually secreted from MAP into the phagosome and then diffused into the cytoplasm of the infected macrophage (Bach et al, 2006). Similarly, the protein kinase G (**PknG**) is secreted within macrophages following uptake, gaining access to the infected macrophage's cytosol. It acts in blocking phagosome-lysosome fusion, thereby preventing the destruction of the pathogen by the lysosomes. This inhibition has been reported in other mycobacteria, such as *M. tuberculosis*, *M. bovis*, and *M. smegmatis* (Walburger et al., 2004).

Traditionally, enzyme linked immunosorbent assays (**ELISA**) are a preferred test to diagnose Johne's disease, mainly due to their benefits of time, cost, and technical ease. However, for preclinical cows that may not be producing sufficient antibodies yet and may still be low fecal shedders of the bacteria, serum ELISA has been shown to have poor sensitivity of less than 30% and specificity less than 100% (Collins et al., 2006; Tiwari et al., 2006). Additionally, the cell-mediated interferon gamma assay, that measures IFN-γ in serum, utilizes specific antigens like protein-purified derivative

Johnin. This assay identifies the host's T cell recognition of antigens since the animals may not have been infected long enough to produce antibodies to MAP. With the potentiating effect of interleukin 12 (**IL-12**) added to the stimulation agent, a greater sensitivity could possibly be achieved (Jungersen et al., 2005; Mikkelson et al., 2009). The efficiency of both types of assays depends on the stage of infection. In addition, for ELISA, the type of immunoglobulin (**Ig**) G targeted is also important, as IgG2 predominates in pro-inflammatory immunity and is therefore indicative of early stage MAP infection or exposure with potential MAP infection. A switch to IgG1 occurs as humoral immunity takes over when cell-mediated control is lost (Koets et al., 2001; Sweeney, 2011). In comparison to antibody ELISAs, the IFN-γ assay performed significantly better in cows less than three years of age (Huda et al., 2004; Jungersen et al., 2012). However, responses with either assay can vary in infected cattle, and IFN-γ detection in exposed animals does not necessarily mean infection will ensue as the cell-mediated immunity may control the infection or the IFN-γ may be only detecting environmental mycobacteria (Jungersen et al., 2002; Huda et al., 2004; Jungersen et al., 2012). In order to address the last issue, the use of early MAP-specific proteins as antigen stimulation was assessed in our study, not only for the IFN-γ assay but also for a novel ELISA that could be used earlier than conventional ELISAs. Gurung et al. (2014) found a higher humoral immune response to PtpA in serum samples from MAP-infected sheep as compared to non-infected sheep, and found that PtpA was secreted throughout the stages of Johne's disease in sheep.

Because Bach et al. (2011) reported that the novel ELISA utilizing PtpA was more sensitive in serum of preclinical cows than a commercial antibody ELISA, we

hypothesized that (1) the novel ELISA would also be sensitive within milk and colostrum samples for early MAP detection due to an increase in immune cells within colostrum and early-lactation milk; and (2) these specific MAP proteins, namely PtpA and PknG, could be used as a sensitive alternative to Johnin in IFN-γ assays, as Johnin has historically shown quite variable activity.

5.3 Materials and Methods

5.3.1 Farm and Cow Selection

5.3.1.1 Novel ELISA

For the development of the novel ELISA, we purposively selected seven Johne's infected dairy farms, from a list comprised in a companion project (Lavers et al., 2013), with three farms from Prince Edward Island, and four from New Brunswick, Canada. MAP prevalence in these selected herds ranged from 3% to 15% as determined in the companion project through individual cow testing using fecal broth culture confirmed by acid fast stain and direct polymerase chain reaction (C. Lavers; University of Prince Edward Island, Charlottetown, PE, Canada, personal communication). Thirty-six MAP-infectious cows were identified for colostrum sampling (as described in Chapter 3) and 48 MAP-infectious Holstein cows were recruited for milk sampling (as described in Chapter 4), from herds ranging between 83 to 490 cows per herd, with a total of 2 to 15 cows recruited per farm. Furthermore, a control group of 52 test negative herdmates was selected from the same farms (as described in Chapter 4). Inclusion criteria for the control group included a history of MAP-negative status on all fecal broth culture, fecal

real-time polymerase chain reaction, milk ELISA, and serum ELISA from the companion project.

As described in the concurrent studies, the target condition was MAP-infectious. The case definition was a dairy cow that had detectable fecal MAP-shedding at least once during a one year period prior to the start of this study. The term MAP-infectious will continue to be used in the remainder of the text to describe these cows. For the control group, the target condition was a MAP test-negative herd-mate. The control group case definition was a herd-mate, matched as closely as possible by age, parity, DIM, and reproductive status to a MAP-infectious cow. These cows were previously confirmed in the companion project (Lavers et al., 2013) as test-negative through fecal broth culture (confirmed with acid fast stain and qPCR), milk ELISA, and serum ELISA.

Because these test-negative cows came from MAP positive farms, they may have been exposed to MAP and therefore not truly negative, despite rigorous testing, but possibly very early preclinical cows. Since the novel tests under evaluation in the current study target very early stage Johne's disease, another group of 47 cows from a consistently test-negative herd from Prince Edward Island was also selected for another negative control group.

In addition, from a known test positive herd in Prince Edward Island, five strongly positive cows were identified for re-sampling from which a pooled sample could be created for a known positive control for the novel ELISA.

5.3.1.2 Whole-blood IFN-γ Release Assay

For the pilot IFN-γ study, four cows from two Prince Edward Island farms were identified. Two of these cows came from a consistently MAP test-negative herd as determined from the companion project (Lavers et al., 2013) and from the Atlantic Johne's Disease Initiative study. The other two cows came from a MAP test-positive farm and had a history of moderate to high milk ELISA scores in the previous study.

5.3.2 Sample Collection

All sample collection protocols were first approved by the Animal Care Committee at the University of Prince Edward Island, Canada.

5.3.2.1 Novel ELISA

Subsequent to cow identification, milk samples were collected monthly from the test and control groups either by the project personnel or by the farmers from July 2010 to December 2011, for a period of up to 12 months for each cow or for as long as the cow remained in the herd. Clean milk samples were taken either between or at regular milking times. Colostrum samples were collected by the farmer within 24 hours of the cow freshening. All milk samples were collected by the farmers the day prior to pick up and all colostrum samples were frozen on farm at -20^{o}C until arrangements could be made for pick-up. Samples were transported on ice to the Maritime Quality Milk Laboratory (**MQM**; Charlottetown, Prince Edward Island), where all milk and colostrum samples were frozen at -80^{o}C until processing.

In addition to milk and colostrum samples, serum samples were collected once at the end of the sampling regime on all remaining cows in the test and control groups.

Whole blood was collected via coccygeal venipuncture into sterile, non-heparinized vacutainer tubes (10 ml; Kendall Monoject™ Blood collection tubes, Tyco Healthcare group LP, Mansfield, MA). Upon arrival at the laboratory, samples were centrifuged at 1,500 x *g* for 10 minutes at room temperature. Subsequently, serum was harvested and stored at -20^{o}C until processing or followed by long term storage at -80^{o}C.

Serum and milk samples were collected from the positive and negative control groups following the same procedures as above. From these, two strongly MAP positive samples were pooled for a positive control reservoir, while four confirmed negative samples were pooled for a negative control reservoir. This was done to address the issues observed during early experiments using reconstituted commercial skim-milk powder (Carnation®, Nestlé, Vevey, Switzerland) as a negative control for milk samples and fetal calf serum for serum samples.

For milk and colostrum samples, 1.8 ml aliquots were shipped to Dr. Horacio Bach's laboratory at the University of British Columbia (**UBC**) for processing, as well as between 0.5 to 1.8 ml aliquots of serum samples, depending on availability.

5.3.2.2 Whole-blood IFN-γ Release Assay

Whole blood samples were collected from the four cows for the IFN-γ study following the same procedure as above, except that approximately 30 ml whole blood was collected in sterile, heparinized vacutainer tubes (10 ml; BD Vacutainer® Lithium Heparin^{N}, Becton Dickinson, Franklin Lakes, NJ, USA). These samples were then transported to the laboratory at ambient temperature, and all samples per cow were

pooled into respective 50 ml conical tubes, then immediately processed following the stimulation protocols as described below.

5.3.3 Laboratory Procedures

5.3.3.1 Novel ELISA

The following protocol was executed at the MQM Laboratory. The same protocol was also used in Dr. Bach's laboratory at UBC, unless noted otherwise. Initially, 25 plates were supplied, pre-coated with recombinant PtpA antigen (as per previously described PtpA production methods in Bach et al., 2006) from Dr. Horacio Bach's Laboratory at UBC, as the antigen itself can become unstable during shipping. These plates were coated with a concentration of 1 μg/μl of PtpA. The total volume of coating was 50 μl. Two other sets of five plates were also sent, one set with 0.5 μg/μl PtpA in the coating, and the other set with 0.25 μg/μl PtpA in the coating. For these plates, the total volume of coating per plate was 100 μl. Another trial was also performed, changing the plate type from cell culture plates, which are not typically recommended for ELISA protocols as they bind larger weight molecules, to ELISA plates (Costar® EIA/RIA flat bottom 96-well plates, Corning Incorporated, Corning, NY). This protocol was also performed separately using PknG and lipase-like proteins in the antigen coating.

Briefly, milk and control samples were diluted 1:1 in phosphate buffered saline (**PBS**) with 3% bovine serum albumin (MQM: Rockland Immunochemicals, Inc., Gilbertsville, PA; UBC: Thermo Fisher Scientific, Waltham, MA). Fifty μl of the diluted sample (from 1 ml solution) and controls incubated for 2 hours at room temperature and

then washed 3 times with PBS-0.05% Tween 20, using the BioTek® ELx 405 ELISA Washer (BioTek Instruments, Winooski, VT, USA) in which the last wash of the cycle was left to soak for 2 minutes. Following, 50 µl Peroxidase-conjugated AffiniPure Goat Anti-Bovine IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added to each well, and the plate was incubated again for 1 hour at room temperature, followed by 3 washes as before. Then 50 µl of 3,3',5,5'-tetramethylbenzidine developer (Pourquier ELISA, Institut Pourquier, France) was added to each well, and the plate incubated for 7 minutes before 25 µl of stop solution (Pourquier) was added to each well. The plate was then read at 450 nm absorbance in the BioTek® Power Wave XS ELISA Reader (BioTek Instruments). For serum samples, the above protocol was followed except that 10 µl of serum samples were diluted in 190 µl (UBC: 10 µl of serum samples were diluted in 990 µl) of sample dilution buffer (Pourquier).

Three control methods were used. Initially, only a negative control of reconstituted commercial skim-milk powder for milk and colostrum assays, and fetal calf serum for serum assays were used. Second, the Pourquier kit controls were tried as well. Last, controls were then switched to the pooled samples of strong MAP positive cows and cows from a test-negative herd. Furthermore, several different dilution ratios of conjugate were used, namely 1:5,000 (with 6 washes), 1:20,000, 1:70,000 (with both 3 washes and 6 washes), 1:85,000 (3 washes), 1:100,000 (3 washes). Furthermore, either duplicate or triplicate runs of the samples were performed and a final value was calculated as an average of the runs minus the negative control. After choosing the best conjugate dilution (1:70,000), an additional method of attempting to reduce plate-to-

plate variability was included. In this method, an alternative final value was calculated from the average of the runs, minus the background value of the coating (a blank well), minus the negative control. Removal of background values from coated blank wells and negative control values was done to adjust for the possibility of IgG concentration within bovine serum albumin and dry milk powder, and to compensate for coating differences between plates.

5.3.3.2 Whole-blood IFN-γ Release Assay

For this pilot study, the response to several stimulation agents for IFN-γ production analysis was assessed. For positive stimulation agent controls, the nonspecific mitogens pokeweed (**PWM**; Sigma-Aldrich, St. Louis, MO, USA) or concanavalin A (**ConA**; Sigma-Aldrich, St. Louis, MO, USA), both in combination with recombinant bovine interleukin-12 p40 (**IL-12 p40**; KingfisherBiotech, St. Paμl, MN, USA) were used. Phosphate buffered saline (pH 7.4) was used as negative control. Against these controls, we assessed the traditional MAP-specific protein purified derivative Johnin (Brucella & Mycobacterium Reagents Team, National Veterinary Services Laboratory, USDA, Ames, IA), Johnin with IL-12 p40, and the novel recombinant proteins PtpA and PknG alone or potentiated with IL-12 p40. Table 5.1 describes the antigens used. Stimulation concentrations for PWM, ConA, IL-12 p40, and Johnin were selected based on the studies of Stabel (1996), Jungersen et al. (2005), and Mikkelsen et al. (2009). For the endotoxin-purified recombinant proteins, three different protein samples were sent to the MQM laboratory post lyophilisation in order to avoid precipitation. Upon arrival, the proteins were reconstituted in 0.5 ml PBS. It was

suspected that some of the protein was lost during lyophilisation. Consequently, after reconstitution, protein concentration was determined using the Bradford standard protocol (Bradford, 1976) as 39.6 μg/ml PknG, 43.8 μg/ml PtpA, and 138.5 μg/ml PtpA. All of the proteins were tested alone and potentiated with IL-12 p40. Unfortunately, due to the loss in concentration from expected values, less protein was available for testing than expected. Therefore, we were only able to test PknG at a concentration of 2 μg/ml and PtpA at concentrations of 2 μg/ml and 5 μg/ml. The amounts of stimulation agents that were available are depicted in Table 5.1.

The procedure for IFN-γ detection via ELISA followed a protocol assessed previously in our laboratory (data not shown). Briefly, 1 ml of whole blood from each cow was added to each of 11 wells per cow in flat-bottom 24-well tissue culture plates (Corning Incorporated, Corning, NY). Then to each respective well per cow, the following room-temperature solutions were added: 2 μl PBS, 10 μg/ml ConA + 10 U/ml IL-12 p40, 2 μg/ml PknG, 2μg/ml PknG + 10 U/ml IL-12 p40, 2 μg/ml PtpA, 2μg/ml PtpA + 10 U/ml IL-12 p40, 5 μg/ml PtpA, 5 μg/ml PtpA + 10 U/ml IL-12 p40, 10 μg/ml Johnin, 10 μg/ml Johnin+10 U/ml IL-12 p40, and 10 μg/ml PWM + 10 U/ml IL-12 p40. After a minimum of 18 hour incubation (overnight) at 37˚C in 5% CO_2, the stimulated blood was transferred to 2 ml microcentrifuge tubes and centrifuged at 500 x *g* for 10 minutes at room temperature. Supernatants were kept in new microcentrifuge tubes at -20˚C until ELISA testing.

To assess IFN-γ production, a commercial sandwich ELISA (ID Screen® Ruminant IFN-γ kit, IDVET, Montpellier, France) was used following the kit protocol. Briefly, to the first 4 wells of a 96-well plate, 25 μl of Dilution Buffer 1 with 25 μl of

negative control and 25 µl of Dilution Buffer 1 with 25 µl of positive control were added to two wells each respectively. To each of the remaining wells, 90 µl of Dilution Buffer 1 and 10 µl of sample were added. All wells were then transferred to an ELISA microplate coated with an anti-ruminant IFN-γ monoclonal antibody (IDVET). After agitation, the plate was incubated for 1 hour at 36^{o}C, followed by 6 washes with 1x wash solution (20x wash concentrate (IDVET) mixed with 19 parts distilled water) in the BioTek® ELx 405 ELISA Washer (BioTek Instruments) to remove unbound proteins. For the antibody-antigen-secondary antibody complex, 100 µl of 1x Anti-ruminant IFN-γ Concentrated HRP Conjugate (10x stock conjugate diluted 1/10 in Dilution Buffer 1) was added to each well, followed by incubation and washing as previously described. Afterwards, 100 µl of Substrate Solution was added to each well, and the plate was incubated in the dark for 17 minutes at room temperature (21^{o}C). Finally, 100 µl of Stop Solution was added to each well to stop the reaction, and the absorbance read at 450 nm in the BioTek® Power Wave XS ELISA Reader (BioTek Instruments). According to the manufacturer's instructions, the test was valid when the mean positive control was >0.5 OD, with the ratio of the mean values of the positive and negative controls >3. Sample OD results were further interpreted as a sample to positive (**S/P**) ratio. This is the ratio of IFN-γ concentration to the positive control and calculated as: S/P (%) = [(OD activated sample – OD non-activated sample)/(OD mean positive control – OD mean negative control)]*100. Samples stimulated with PBS were used to calculate OD control. Positive cut-off for IFN-γ production required an S/P ratio >15%.

5.3.4 Statistical Analysis

Statistical analysis was done using STATA® 12 (StataCorp LP, College Station, Texas, USA) and Excel 2010 (Microsoft, Mississauga, Ontario). Statistical significance was set at $P < 0.05$.

For the novel ELISA, mean, median, and standard deviations were calculated on the final values from the three different sample types. Analyses for possible diagnostic utility of the novel ELISA were assessed via receiver operating curves (**ROC**) and cut point selections for best sensitivity and specificity outcomes (Dohoo et al., 2009).

For the novel IFN-γ assay, mean, median, and standard deviations were calculated for infected and non-infected samples across stimulation types. Bar graphs were created to represent these results over infection status (average of cows per group) and also per cow to depict differences between cows. Due to the small sample size of this pilot study, the results focused on descriptive and univariate analyses.

5.4 Results

5.4.1 Novel ELISA

5.4.1.1 Descriptive Data

For the novel ELISA study, cows ranged from two to nine years (mean = 4.6 years), and from first to seventh parity (mean = 2.8). There were a total of 187 milk, 44 colostrum, and 56 serum samples from infected cows and 208 milk, 8 colostrum, and 42 serum samples from non-infected cows, all from MAP-positive herds. From the MAP-negative herd, 47 milk and serum samples were collected.

Variability was observed for the novel ELISA detecting early expressed PtpA and PknG proteins in both MAP-infected and non-infected cows from MAP test-positive and -negative herds. Results will be discussed in chronological order following the described improvements and variations adopted to the original design and protocol.

5.4.1.2 First Control Set: Only Negative Controls

At the UBC laboratory, positive and negative samples were analyzed initially on separate ELISA plates, and the operator was not blinded to the sample status. Initial results showed high variability in negative control values in the plates containing samples from MAP-infectious cows as compared to those in the plates containing samples from MAP test-negative cows.

As indicated in Table 5.2, there was only a small difference between results for colostrum from known MAP-negative and MAP-positive cows, but a much greater difference overall in milk samples. Ranges showed much overlapping between MAP-negative and MAP-positive sample outcomes for all three sample types.

For the milk samples, a ROC curve analysis revealed an area under the curve of 75.3% and indicated a possible cut-point of 0.3 to detect a positive cow. However, accurate comparison between plates was not possible as plates were segregated between negative and positive samples rather than mixed samples per plate. Furthermore, standard deviations were approximately 30% to 40% larger for positive cow results as compared to those from negative cows.

5.4.1.3 Non-segregated Plates and Strong Positive and Negative Pools for Controls

Modification to the intial experimental design was made to avoid the difficult interpretation of plate-to-plate variation. Each plate included new positive and negative controls (from pooled samples from strong MAP-positive cows and from MAP-test negative cows from a test-negative herd), and with various dilutions of milk. Not much difference was noted between positive and negative milk samples on several trials. After these changes, best results for milk samples from MAP-positive cows had an average OD reading of 0.35, with OD values ranging from 0.047 to 1.945. For negative milk samples, however, the mean OD was 0.111, but the range was from 0.043 to 1.093, overlapping with positive cows.

5.4.1.4 Alternate Coating-protein Concentrations

We then assessed different coating concentrations as described above, but again not much difference was noted between MAP status samples. In general, 1:70,000 was most suitable with the highest positive readings. However, it did result in a negative control value of approximately 0.2 and higher blank well values. Nevertheless, this was corrected for in the final result as previously discussed.

Samples were processed from a MAP test-negative herd as a true negative status and compared against an assortment of MAP positive and negative (exposed) samples from test-positive herds. Table 5.3 shows the results from two separate runs. Comparisons between plates could not be accurately assessed as the positive control we were using up to this point was not effective in these runs.

5.4.1.5 Alternate Type of Plate

We then changed from cell culture plates to true ELISA plates, and we compared our results to an ELISA run that Dr. Bach performed at the UBC laboratory (using the same samples that we used in the MQM laboratory). This was done to compare the protocols, both for milk and serum samples. Results were not improved, as some comparisons were not much different between negative and positive samples. Overall, corrected average OD reading for positive versus negative samples were 0.249 (range: 0.137 to 0.383, standard deviation: 0.74) and 0.168 (range: 0.097 to 0.2345, standard deviation: 0.056), respectively. As a result of so much overlap in the data, ROC curve analysis (data not shown) did not reaveal any meaningful Se and Sp cut point analysis.

5.4.1.6 Alternate Coating-protein

We speculated that the level of PtpA antibodies was too low to be detected by ELISA in colostrum and milk samples. Therefore, we tested new antigen by adsorbed ELISA using the same experimental sample panel. The new antigens included PknG and lipase-like proteins. Table 5.4 shows that milk diluted 1:1 with PBS had inconclusive results. Colostrum results were promising, with positive cows in positive herds giving the highest OD readings, as compared to MAP test-negative cows. However, these were based on single cows in each group.

5.4.2 Whole-blood IFN-γ Release Assay

Interferon gamma was detected with both PknG and PtpA (Figure 5.1), although results were variable among the four cows. In particular, the second negative cow unexpectedly showed very high positive results for samples stimulated with ConA + IL-

12 p40 and PtpA alone and with IL-12 p40 (Figure 5.2). The effect of this may have caused higher than normal average negative-cow results, particularly for PtpA at 2 μg/ml with and without IL-12 p40. Due to missing values, not all categories have results from all four cows, making comparisons between groups difficult. Results for PWM + IL-12 p40 were not included in both figures due to over-expression of IFN-γ. Both PknG + IL-12 p40 and PtpA (5 μg/ml) + IL-12 p40 produced the best results, with a difference of 63.9% and 17.2% S/P points between positive and negative cows, respectively. However, these results came from only one negative and one positive cow. For PtpA at 2 μg/ml concentration, proportions were similar but opposite those for PtpA at 5 μg/ml. Finally, Johnin did not produce detectable IFN-γ at the cut-point designated by the commercial ELISA kit, regardless of MAP status in the cows.

5.5 Discussion

One of MAP's hallmark survival mechanisms is to evade the host's natural immune responses. Earlier infection diagnosis could lead to quicker instigation of specific, efficient control mechanisms (Stabel et al., 2007). However, current diagnostic tests are not reliable for early diagnosis of preclinical cows (Kalis et al., 2003). Therefore, the use of early MAP proteins, such as PknG and PtpA, as specific antigens in diagnostic tests, such as ELISA and IFN-γ, is a step towards developing a rapid, yet sensitive and specific early MAP identification tool. Since MAP proteins (PtpA, PtpB, and PknG) are secreted early in infection (upon phagocytosis) to block part of the

immunological response mounted by host macrophages (Bach et al., 2011), we hypothesized that these proteins can serve as part of an early diagnostic test.

5.5.1 Novel ELISA

It has been reported that IgG levels detected by an antibody-using ELISA are more likely to result in positive MAP detection during late lactation for serum and during either early or late lactation for milk of MAP-infected cows (Nielsen et al., 2002; Lombard et al., 2006). Colostrum samples and the first few days of milk production are suspected to have high levels of both IgG1 (humoral) and IgG2 (pro-inflammatory); and also the milk is less diluted at early and late lactation, resulting in a higher concentration of immunoglobulins and MAP antigen (Nielsen and Toft, 2012). Actually, the IgG2 concentration in colostrum has been reported to be at a minimum of 30-fold increase compared to milk (Zervens et al., 2013). Therefore, the use of not only an ELISA for colostrum or early milk samples but also an ELISA that can be used for preclinical, early infection identification would be ideal. Our study aimed at addressing both these conditions through the use of early secreted proteins, like PtpA, in humoral immunity assays. With the novel protein acting as antigen, the ELISA therefore measures the presence of antibodies specific to these proteins. Although milk samples in early lactation could be more likely to identify IgG2 antibodies, the possibility of false positive results also exists. This possibility lies in the higher concentration of nonspecific proteins in these early lactation samples that could be falsely identified as a bound antibody in the ELISA (Nielsen and Toft, 2012). Nielsen and Toft (2012) recommend long-term follow-up, and suggest that the same response should be seen in

either high or low prevalence herds when there is nonspecific protein binding. However, Zervens et al. (2013) found that nonspecific ELISA reactions in colostrum were actually very small (3/365 samples) and that colostrum from the day of parturition was 130 times more likely to exhibit a positive response to an indirect commercial ELISA compared to milk samples from cows only four days in milk.

In many of our runs, too much variation in negative controls made for too much uncertainty in validity of sample results. Initially, it is possible that there was some cross-contamination in positive non-segregated plates or overall that coating was variable. Another possibility lies in the fact that some of the negative cows may have had higher values because they were exposed cows from a MAP test-positive farm. Although our initial goals focused on the use of the test in colostrum only, the preliminary data shown in Table 5.2 indicates poor results for colostrum at this time, while results for milk were more promising. Although the last assays using PknG and lipase-like proteins showed potentially applicable results for colostrum in the future (Table 5.4), with regards to the milk ELISA protocol, these results may have been sub-optimal as Dr. Bach often detected precipitation at the bottom of the wells using a 1:1 milk dilution. Despite adjusting OD values by accounting for background values from the coating, the results may have misrepresented the actual protein concentration. Consequently, further analysis is currently being pursued.

A large portion of the development for the novel ELISA included looking at different concentrations of antigen-protein coating, different dilution ratios, different negative controls, as well as use of negative cows from a MAP test-negative herd. Table 5.3 showed results from samples from a test-negative herd. Some of the duplicates in

these runs were highly variable, which raised concerns about a possible issue with uneven coating across the plate, or perhaps somewhat partially desiccated coating during shipment and storage. Unfortunately, despite Dr. Bach's attempts at overexpression of all three proteins, yield was very low and slow, making repeated attempts within a short period of time for a large amount of samples difficult. Therefore, further work is also being assessed for greater and more efficient *in vitro* production of these virulence proteins to enhance diagnostic test development assays.

5.5.2 Whole-blood IFN-γ Release Assay

By sensitizing the recall mechanism of lymphocytes through the action of specific stimulation agents, IFN-γ is produced and can be detected through cell-mediated assays. Although detection of specific antibodies via ELISA is ideal for identification of early infection or preclinical animals, detection of IFN-γ, although not necessarily always indicating infection, identifies exposure and thereby transmission occurring in the herd (Zervens et al., 2013). Since cell-mediated responses are strong during early infection, IFN-γ testing can be used as a support mechanism to identify gaps in Johne's disease management protocols that could cause MAP exposure, especially for preclinical cows and calves (Collins, 1996; Kalis et al., 2003). In contrast, antibodies used in ELISAs are not efficient for cows under three years of age due to the lack of detectable humoral immune activity (Huda et al., 2004). Furthermore, antibodies may be at a low concentration during earlier stages of infection (Jungersen et al., 2012). Traditionally, the IFN-γ assay, using Johnin, is known for its low specificity (Mikkelsen et al., 2009) and high detection variability in young calves, particularly up to six months of age, with

better sensitivity for one to two year olds (Jungersen et al., 2002; Huda et al., 2003, 2004; Jungersen et al., 2012). Our goal was to determine if the virulence proteins could be as effective as or even better than the effects of other protein purified derivatives, such as Johnin, for infected cows in comparison to non-infected cows.

In our analysis, there was a lot of variation, unfortunately, between the two noninfected and two infected cows (Figure 5.1). Nevertheless, the strong response to ConA + IL-12 p40 indicated that viable lymphocytes were present in our samples and that there were no false negative reactors present because the non-stimulated samples did not show an increased IFN-γ level (Stabel and Whitlock, 2001). The lower response to ConA in the second MAP-infected cow (Figure 5.2) may be due to a waning cell-mediated response known to occur in clinical cows (Stabel and Whitlock, 2001), although the clinical status of this cow was not recorded at the time of sampling. Even though degree of infection was not recorded for the positive cows, they were milk ELISA positive prior to this study. In preclinical Johne's infected cows, Stabel et al. (2007) noted an increased IFN-γ response to ConA-stimulated blood as compared to PWM-stimulated blood following pre-sensitization with Johnin intradermally. However, as also seen in our study, Jungersen (2002) suggested that ConA may have a reduced effect when there is a positive humoral response to a mycobacterial antigen and during clinical phases of the disease. Verschoor et al. (2010) had also reported inconsistent assay results during clinical paratuberculosis.

For both PtpA and PknG, IFN-γ was detected, with somewhat better results for PknG (Figure 5.1). Potentiation with IL-12 p40 increased the level of IFN-γ detection. Jungersen et al. (2005) warns that IL-12, although able to help rescue a weak immune

response, could potentially result in false positive results by simultaneously stimulating natural killer cells, rather than just lymphocytes, to produce IFN-γ, despite the fact that natural killer cells can also have memory function of previous disease exposure. There is a higher prevalence of natural killer cells in calves, therefore increasing a risk of false positives in younger cattle (Kulberg et al., 2004) especially those <15 months of age (Jungersen et al., 2002). In our study, we obtained blood samples from adult cows, and therefore the risk of false positives may be low, but the exact effect is unknown without concurrent analysis of the blood samples for proportion of specific immune cells present at the time of stimulation. Kalis et al. (2003), however, did not find an association between IFN-γ skin test assay specificity and age.

It was unclear as to why PtpA at a 2 μg/ml concentration would detect more IFN-γ than at 5 μg/ml (Figure 5.1), with PtpA at 5 μg/ml producing only borderline results at the positive cut-off for IFN-γ detection. Also Johnin was least sensitive, but there was concern about the viability of the antigen batch within our laboratory, and it would be recommended to repeat the test with a new batch of Johnin antigen before speculating on these results. Nielsen and Toft (2008) have reported that for infectious cows, the sensitivity of IFN-γ assays varied between 13% and 85%, with specificity ranging between 88% and 94%. It has also been noted that greater than three year old cows or cows already in the humoral immunity status or even in the clinical stage of Johne's disease likely show a reduced response to Johnin (Mikkelsen et al., 2009). This may have occurred in our study as we did not have any youngstock against which to compare the results. Furthermore, high variability within Johnin preparations from

various potencies and different source strains to a lack of standardization in its production makes comparison among studies difficult (Bannantine et al., 2010).

5.5.3 Conclusion

Although our results are still preliminary and not yet applicable for diagnostic test development, results indicated that PtpA and PknG, early MAP-virulence proteins, are detected as MAP antigens in milk, colostrum, and serum samples of MAP-infected cows with both ELISA and IFN-γ assays. The identification of highly specific antigens for MAP for either improved IFN-γ assay or a preclinical ELISA are imperative for developing a preclinical MAP-infection identification test or a highly reliable early-use decision-support assay for MAP control that can be used for calves or preclinical cows. Further work in analyzing these virulence proteins in not only infectious cows but also suspected exposed cows and calves from test-positive herds in comparison to test-negative herds is underway. Further analyses include improvements to ELISA coating applications, including protein concentration and combinations to improve sensitivity and specificity of the assay, as well as improvements to the IFN-γ assay using larger sample sizes and samples from heifers and calves.

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Table 5.1. Volume (microliters) of stimulation agents and negative and positive controls added to whole blood samples from two *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infected cows and two non-infected cows.

	Stimulation Agents[b]										
		Positive Controls		PknG		PtpA				Johnin	
Cow[a]	PBS	A[c]	B[c]	C	D[c]	E	F[c]	G	H[c]	I	J[c]
N	2	2	10	52	52	14.4	14.4	113	36	10	10
N	2	2	10	0	0	14.4	14.4	0	0	10	10
P	2	2	10	52	52	14.4	14.4	113	36	10	10
P	2	2	10	<52	0	14.4	14.4	<100	0	10	10

[a]N = cows from a MAP-test-negative farm; P = cows with positive milk ELISA results from a MAP test positive farm.
[b]PBS = phosphate buffered saline as negative control; Positive controls (A = ConcanavalinA + interleukin-12p40, B = Pokeweed + interleukin-12p40); PknG = protein kinase G (C = PknG, D = Pkn G + interleukin-12p40); PtpA = protein tyrosine phosphatase A (E = PtpA at 2 μg/ml, F = PtpA at 2 μg/ml + interleukin-12p40, G = PtpA at 5 μg/ml, H = PtpA at 5 μg/ml + interleukin-12p40); Johnin purified protein derivative (I = Johnin, J = Johnin + interleukin-12p40).
[c]Samples from each cow in these columns had four microliters of interleukin-12 p40 added to the listed volume of stimulation agent. When no stimulation agent was available for a sample, four microliters of interleukin-12 p40 alone was added.

Table 5.2. Quantification [a], by an adsorbed enzyme linked immunosorbent assay, of the presence of protein phosphatase A antibodies in milk, colostrum, and serum samples from known *Mycobacterium avium* subspecies *paratuberculosis* (MAP)-infected and non-infected cows.

	MAP status	Total observations	Mean OD	Median OD	Range OD	Standard Deviation
Milk	Negative	208	0.028	0.002	(-0.075, 1.035)	0.090
	Positive	187	0.240	0.082	(-0.061, 1.861)	0.383
Colostrum	Negative	8	0.097	0.081	(-0.014, 0.234)	0.092
	Positive	44	0.103	0.036	(-0.043, 0.701)	0.158
Serum	Negative	42	0.053	0.001	(-0.018, 1.362)	0.214
	Positive	56	0.100	0.046	(-0.018, 0.585)	0.130

[a] Mean, median, range, and standard deviation of average enzyme linked immunosorbent assay optical density (OD) values from triplicate runs minus the negative control per plate (coated with MAP protein tyrosine phosphatase A antigen)

Table 5.3. Corrected mean optical density (OD) readings[a] from a *Mycobacterium avium* subspecies *paratuberculosis* (MAP) protein tyrosine phosphatase A antigen-coated enzyme linked immunosorbent assay for milk samples from a MAP test-negative herd as well as from infected and non-infected (exposed) cows from MAP test-positive herds.

Status[b]	Total observations	Run	Corrected mean OD
TN herd	40	1	0.20
TP herd, positive	23	1	0.225
TP herd, negative	22	1	0.20
TN herd	54	2	0.08
TP herd, positive	16	2	0.17
TP herd, negative	20	2	0.008

[a][raw value minus the background (blank-coated well) and negative control values]
[b]TN = MAP test-negative herd; TP = MAP test-positive herd from which there were positive (infected) and negative (non-infected) cows.

Table 5.4. Quantification [a], by an adsorbed enzyme linked immunosorbent assay, of the presence of protein kinase G and lipase-like protein antigens for milk and colostrum samples from known *Mycobacterium avium* subspecies *paratuberculosis* (MAP)-infected and non-infected cows.

	MAP status		Total observations	Mean OD	Median OD	Standard Deviation
	Cow	Herd				
Milk	Negative	Negative	10	0.029	0.008	0.040
	Positive	Positive	15	0.019	0.023	0.018
Colostrum	Negative	Negative	1	0.170	0.170	n/a
	Positive	Positive	1	0.764	0.764	n/a
	Negative	Positive	13	0.271	0.247	0.177

[a] Mean, median, and standard deviation of average optical density (OD) values from duplicate runs minus the background (blank-coated well) value from the adsorbed enzyme linked immunosorbent assay

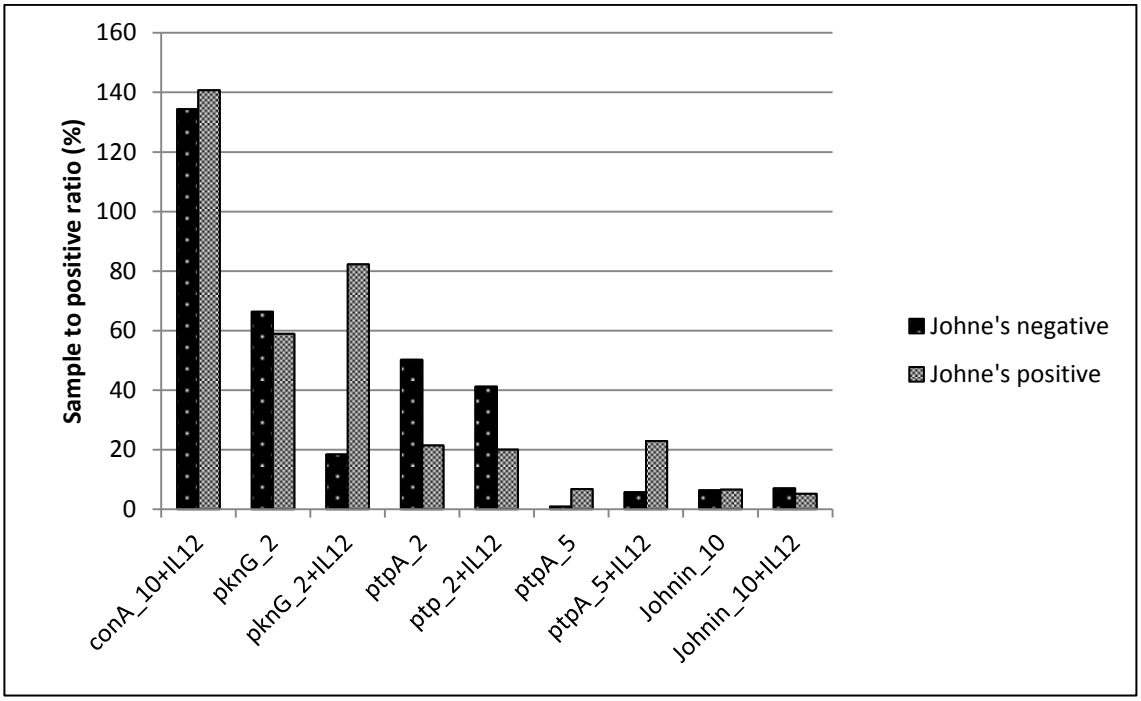

Figure 5.1. *In vitro* interferon gamma production measured by whole-blood assay, exposed to different antigens or stimulation agents[a], from two Johne's infected and two non-infected cows.

[a]Stimulation agents: nonspecific mitogen concanavalinA (conA) at 10 µg/ml potentiated with interleukin-12p40 (IL12) at 10 U/ml; protein kinase G (pknG) at 2 µg/ml alone and with IL-12 p40; protein tyrosine phosphatase A (ptpA) at either 2 µg/ml or 5 µg/ml alone and with IL-12 p40; and protein purified derivative Johnin at 10 µg/ml alone and with IL-12 p40.

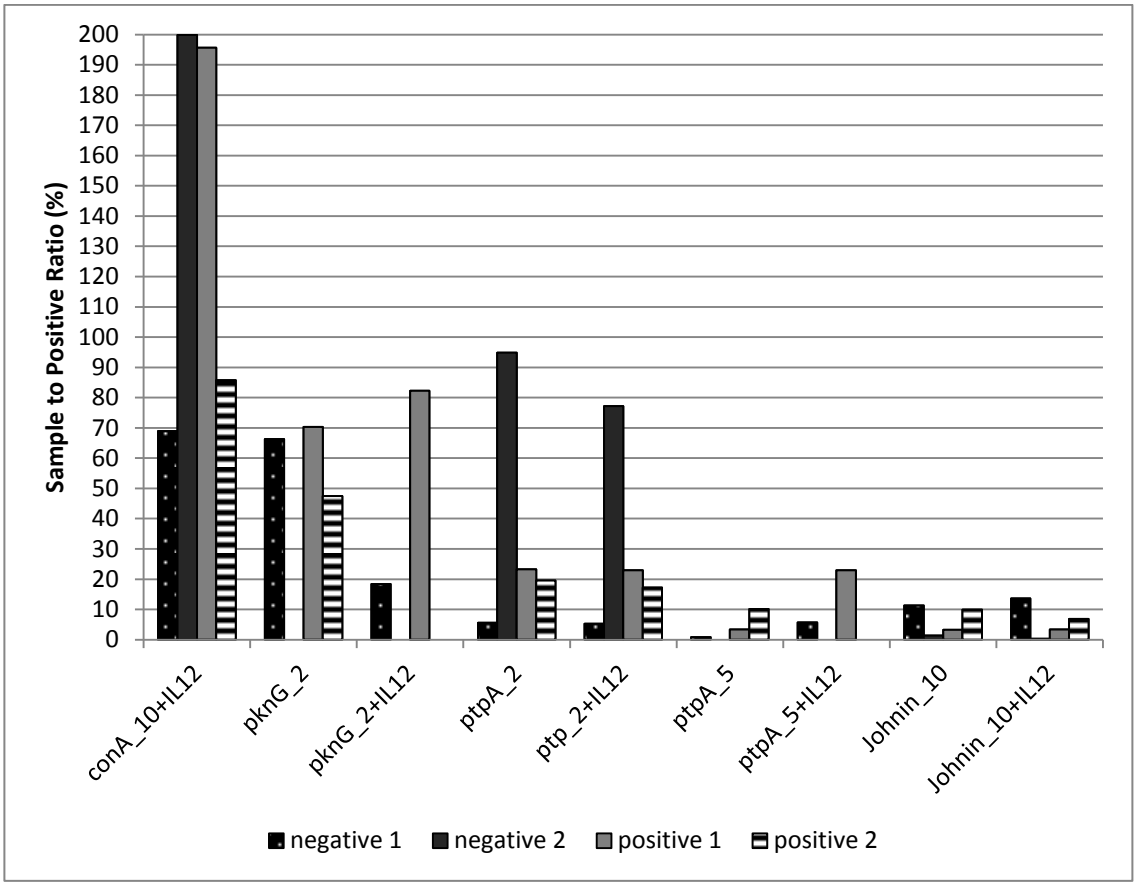

Figure 5.2. Interferon gamma production as measured by sample to positive ratio percent for whole blood samples, from two Johne's infected and two non-infected cows, which were treated with nine stimulation agent variations[a].

[a]Stimulation agents: nonspecific mitogen concanavalinA (conA) at 10 µg/ml potentiated with interleukin-12p40 (IL12) at 10 U/ml; protein kinase G (pknG) at 2 µg/ml alone and with IL-12 p40; protein tyrosine phosphatase A (ptpA) at either 2 µg/ml or 5 µg/ml alone and with IL-12 p40; and protein purified derivative Johnin at 10 µg/ml alone and with IL-12 p40.

CHAPTER 6

NOVEL CELL PRESERVATION TECHNIQUE TO EXTEND *IN VITRO* WHITE BLOOD CELL VIABILITY REQUIRED FOR CELL-MEDIATED DIAGNOSTICS IN DAIRY COWS.

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The primary contents of this chapter have been submitted to The Veterinary Journal.

6.1 Abstract

Cell-mediated immunity based diagnostics, particularly assays for pro-inflammatory cytokines such as interferon gamma (IFN-γ), can be used for early detection of Johne's disease. However, traditionally the IFN-γ test requires blood sample processing within 24 hours of collection to maintain white blood cell (WBC) viability. Therefore, to improve the utility of this test, the objective of this study was to assess the use of a novel WBC preservation technology in whole bovine blood. Blood samples from ten healthy cows were divided into an unpreserved control sample and a test sample preserved with transport medium (SCSR-T™, NonInvasive Technologies). Samples were maintained at room temperature and stimulated with the mitogens pokeweed or concanavalinA (ConA), as well as with interleukin-12 (IL-12 p40). Stimulation was completed on days 1, 5, and 8 post-sampling. Viability of WBCs was assessed through IFN-γ production determined with a commercial enzyme linked immunosorbent assay (ID Screen®, IDVET). In addition, mononuclear cell viability was assessed with propidium iodide flow cytometry. Peservation allowed for higher IFN-γ detection in ConA or ConA+IL-12 p40 stimulated blood days 5 and 8 post-collection. Additionally, viable mononuclear cells were still present at 8 days post-collection, with a significantly higher mean proportion detected at days 5 and 8 in preserved samples. This practical and simple method to extend *in vitro* WBC viability could allow for more efficient utilization of cell-based blood tests for Johne's disease diagnostic and control programs.

6.2 Introduction

Cell-mediated blood tests are sensitive tests for Johne's disease diagnostics and control by more readily detecting preclinically infected animals when early immune responses prevail (Stabel, 1996). The first stage of infection with *Mycobacterium avium* subsp. *paratuberculosis* (**MAP**), the bacterial etiology for Johne's disease or paratuberculosis, activates the innate immune system and adaptive immunity, particularly cell-mediated immunity, in susceptible hosts. As a chronic, production limiting disease in dairy cows, Johne's disease is known for the hallmark ability of MAP to infect, reside and multiply within host macrophages (intracytoplasmatic infection) by avoiding and altering normal pathways of host immunity to clear the infection (Bannantine and Stabel, 2002).

During the initial stages of infection, when antigen-presenting cells capture and process pathogens, a cell-mediated response is activated. During the pro-inflammatory stage of the disease, pro-inflammatory cytokines, such as interleukin (**IL**)-12, are released from those cells, polarizing naïve Th0 cells into Th1 cells that secrete interferon gamma (**IFN-γ**) and activate macrophages (Stabel 1996; Stabel, 2010). In addition, the presence of IFN-γ induces the production of immunoglobulin (**Ig**) isotype G2 and inhibits the secretion of IgG1 from activated plasma B cells. As the incubation period for the disease progresses and an anti-inflammatory state unfolds, the prevalence of anti-inflammatory cytokines (for example, IL-10) leads to suppression of IFN-γ production

and a subsequent humoral response with the secretion of IgG1 antibodies (Nielsen and Toft, 2006; Stabel, 2010; Sweeney, 2011).

Detection of this cell-mediated response to bacterial invasion in exposed and infected animals (Stabel, 1996) can be a practical tool to aid early control measures. The IFN-γ assay is a diagnostic tool for cell-mediated detection of Johne's disease, measuring the animal's exposure to MAP more effectively than humoral antibody ELISA (Stabel and Whitlock, 2001; Nielsen and Toft, 2006). However, traditionally, this test requires processing of bovine blood samples within 24 hours of collection in order to maintain white blood cell (**WBC**) viability (Plain et al., 2012). Consequently, this test has never gained widespread use in Johne's disease diagnostics, despite the benefits of the IFN-γ assay.

Because of challenges in identifying animals early in the MAP infection process, this study evaluated nonspecific mitogen effects on IFN-γ production with and without addition of a novel cell transport media. Stabel (1996) has reported similar IFN-γ responses with mitogen-stimulated blood samples from both non-infected and sub-clinically infected cows. Therefore, nonspecific mitogens can be used to stimulate up to 90% of lymphocyte blastogenic response, eliciting IFN-γ production regardless of MAP status. Commonly used mitogens, such as pokeweed (**PWM**) and concanavalinA (**ConA**), stimulate primarily B cells or T cells, respectively, and can thereby be used for general differentiation (Stabel, 1996; Gershwin et al., 2005, p. 105). Furthermore, the Th1 cytokine IL-12 promotes survival and growth of Th1 immunity, sustaining efficient numbers of memory or effector Th2 cells but inhibiting the formation of Th2 immunity. This cytokine can be used synergistically with mitogens due to its immunostimulatory

effects on T cells and natural killer (NK) cells to secrete IFN-γ (Vignali and Kuchroo, 2012; Yim et al., 2013). Jungersen et al. (2002) stressed that although viable T cells and successful antigen presentation typically occurs within eight hours post-collection, IL-12 potentiation could allow this time interval to be extended to 24 hours (Jungersen et al., 2005).

In addition to assessing cell viability through IFN-γ production, the presence of specific viable WBCs in a blood sample can be determined via flow cytometric analysis. It is expected that *in vitro*, T cells have a half-life of up to two days (Plain et al., 2012). In a study of *Mycobacterium bovis* stimulated blood, the amount of WBCs isolated decreased from fresh to two days old blood at temperatures greater or less than room temperature (Senogles et al., 1978). It is for this reason that cell-mediated blood tests have held to the protocol of processing samples within 24 hours of collection (Senogles et al., 1978). Indeed, Robbe-Austerman et al. (2006) recommended that whole blood kept at room temperature should be processed within 12 hours.

Therefore, the goal of our study was to assess the use of a novel blood cell preservation media (SCSR-T™, NonInvasive Technologies) as a practical method of extending the lifespan of WBCs *in vitro* to allow for extended sample transit time. To evaluate this, both nonspecific stimulation of IFN-γ and flow cytometry were subsequently pursued to assess cell viability in preserved and unpreserved samples.

6.3 Materials and Methods

6.3.1 Sample Collection

Animal protocols were approved by the Animal Care Committee at the University of Prince Edward Island before commencement of the study. Ten healthy Holstein dairy cows were selected for participation from a farm in Prince Edward Island, Canada. Approximately 20 ml of blood was collected per cow by tail vein venipuncture into four heparinized vacutainer tubes (BD Vacutainer® Lithium Heparin^{N}, Becton Dickinson, Franklin Lakes, NJ, USA).

6.3.2 Sample Preparation

Immediately upon arrival at the laboratory post-collection, all vacutainer tubes of whole blood samples were pooled into 50 ml polypropylene conical tubes per cow, after which, half of the volume for each was decanted into a second 50 ml conical tube. One tube per set was kept as an unpreserved control, while the whole blood in the other tube was preserved with 1:1 (*v/v*) ratio of transport medium (SCSR-T™ Biological Sample Preservation Medium, NonInvasive Technologies, Elkridge, MD, USA). All 20 tubes were then maintained bench-top at room temperature (21˚C) in the laboratory for eight days during the study.

6.3.3 Stimulation Agents

Stimulation agents for IFN-γ production and release analysis in this study included the mitogen PWM (Sigma-Aldrich, St. Louis, MO, USA), the mitogen ConA (Sigma-Aldrich, St. Louis, MO, USA), recombinant bovine IL-12 p40 (KingfisherBiotech, St. Paul, MN, USA), and a combination of ConA and IL-12 p40.

Phosphate buffered saline (**PBS**; pH 7.4) was used as a negative control in both preserved and unpreserved whole blood samples. Stimulation agent concentrations, as described below, were selected based on the studies of Jungersen et al. (2005), Mikkelsen et al. (2009), and Stabel (1996). Furthermore, results of previous mitogen titration analyses performed in our laboratory (data not shown) guided our choice of dosages for PWM and ConA to avoid over-stimulation.

6.3.4 Stimulation Method

Methodology for transport medium use followed the foundational guidelines from NonInvasive Technologies (SCSR-T™ 2007 Instructions Pamphlet, www.noninvasivetech.com), where the media is used primarily for human intestinal epithelial cells (Nair et al., 2011). However, to facilitate its use for bovine whole blood, we used the following adapted protocol for the IFN-γ production and release analysis for this study. The stimulation was performed on both unpreserved and preserved samples of whole blood on days 0 (collection day), 4, and 7, with cells harvested on days 1, 5, and 8. Briefly, from unpreserved samples 1 ml of whole blood was added to each of 5 wells per cow in flat-bottom 24-well tissue culture plates (Corning Incorporated, Corning, NY, USA). From preserved samples, 2 ml of diluted whole blood in transport medium as mentioned above, was placed into five corresponding 2 ml microcentrifuge tubes per cow and centrifuged at 500 x *g* for 10 minutes at room temperature. The supernatant was discarded, and the remaining pellets were re-suspended with Dubelccos' Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum, to complete 1 ml per tube. The suspension was transferred to each of

another five wells per cow in the 24-well tissue culture plates. Then, to each respective set of two wells per cow (unpreserved whole blood and re-suspended pellet from preserved sample), the following room-temperature solutions were added, for a total of 10 wells per cow: 2 µl PBS, 10 µl (10 µg/ml) PWM, 2 µl (10 µg/ml) ConA, 4 µl (10 U/ml) IL-12 p40, and a combination of 2 µl (10 µg/ml) ConA plus 4 µl (10 U/ml) IL-12 p40. The plates were then incubated overnight (approximately 18 hours) at 37˚C in an atmosphere supplemented with 5% CO_2. After incubation, the content of each well was transferred to a respective 2 ml microcentrifuge tube and centrifuged at 500 x *g* for 10 minutes at room temperature. The supernatants were transferred to new microcentrifuge tubes and frozen at -20˚C until ELISA assessment.

6.3.5 Interferon Gamma Analysis with ELISA

Assessment of IFN-γ concentration per sample was completed after the last stimulation day using a commercial sandwich ELISA (ID Screen® Ruminant IFN-γ kit, IDVET, Montpellier, France), according to the manufacturer's instructions. Briefly, 25 µl of Dilution Buffer 1 and 25 µl of negative control were added to the first two wells of a 96-well plate, followed by 25 µl of Dilution Buffer 1 and 25µl of positive control to the next two wells. Then 90 µl of Dilution Buffer 1 and 10 µl of corresponding samples were added to the remaining wells. All of these were transferred to an ELISA microplate coated with an anti-ruminant IFN-γ monoclonal antibody, and the plate was agitated followed by incubation for one hour at approximately 36˚C. After incubation, the plate was washed six times with 1x wash solution (20x concentrate mixed with 19 parts distilled water) in the BioTek® ELx 405 ELISA Washer (BioTek Instruments, Winooski,

Vt, USA) to remove unbound proteins. Then 100 µl of 1x Anti-ruminant IFN-γ concentrated Horse Radish Peroxidase (**HRP**) Conjugate (10x stock conjugate diluted 1/10 in Dilution Buffer 1) was added to each well to form an antibody-antigen-secondary antibody complex. The secondary antibody is conjugated to the enzyme HRP that can produce a color signal. The plate was then incubated again and washed as before. Then 100 µl of Substrate Solution was added to each well, the plate was incubated for 17 minutes at room temperature in the dark and 100 µl of Stop Solution added to each well to stop the reaction. The plate was then placed in the BioTek® Power Wave XS ELISA Reader (BioTek Instruments, Winooski, VT, USA). The absorbance was read at 450 nm. According to the procedure protocol, the test is valid if the mean optical density (**OD**) of the positive control is >0.5 and the ratio of the mean values of the positive and negative controls is >3. The results for each sample were interpreted as a sample to positive (**S/P**) ratio, or a ratio of IFN-γ concentration to the positive control using the following formula: S/P (%) = [(OD activated sample – OD control sample)/(OD mean positive control – OD mean negative control)]*100. For both the preserved and unpreserved samples, the OD for the control sample was determined from the samples with only PBS added. According to procedure protocol, samples with S/P ratio >15% were considered positive for IFN-γ production.

6.3.6 Cell Viability Analysis with Flow Cytometry

White blood cell viability was assessed via propidium iodide flow cytometry over time (Davey and Kell, 1996). After incubation of samples and removal of supernatant as described above, the remaining pellet was re-suspended in PBS to a final

volume of 1 ml. Then 200 µl of each sample was transferred to respective flow cytometry tubes. To each flow tube, 5 µl (10 µg/ml) propidium iodide (Sigma-Aldrich, Co., St. Louis, MO) in PBS (Thermo Scientific™ HyClone™, Fisher Scientific Co., Ottawa, ON, Canada) and 2 ml of 1x lysis buffer (10 ml of 10x BD Pharm Lyse™ Stock (BD Biosciences, Mississauga, ON, Canada) with 90 ml distilled, deionized water) was added, and the tubes vortexed to immediately lyse the red blood cells (RBCs). By lysing the RBCs, the number of remaining WBCs could be accurately determined. The tubes were then incubated at room temperature in the dark for 15 minutes, followed by centrifugation at 200 x *g* for 5 minutes at room temperature. The supernatant was subsequently aspirated without disturbing the cell pellet. Then 2 ml of 1x PBS with 1% fetal bovine serum (VWR International, Mississauga, ON, Canada) and 0.1% sodium azide (Fisher Scientific Co., Ottawa, ON, Canada) was added to the pellets, which was centrifuged at 200 x *g* for 5 minutes at room temperature, and the supernatants discarded. The pellets were then re-suspended in 0.5 ml of 1x PBS with 2% formaldehyde (Fisher Scientific Co., Ottawa, ON, Canada) to fix the cells, which were then kept at 4˚C until flow cytometric analysis after the last stimulation day.

For flow cytometry, the samples were processed through the BD FACSCalibur™ Flow Cytometer (BD Biosciences, Mississauga, ON, Canada). To calculate cell percentages, 10,000 events per sample were read. Propidium iodide dye is excited at 488 nm. The resultant WBC concentration was gated into respective live and dead mononuclear cell (monocytes, T cells, B cells, and NK cells) percentage and live and dead polymorphonuclear cell (eosinophils, basophils, neutrophils) percentage, as the dye penetrates the cells with damaged membranes (Davey and Kell, 1996).

6.3.7 Statistical Analysis

Statistical analysis was done using STATA® 12 (StataCorp LP, College Station, Texas, USA) and SAS (SAS Institute Inc., Cary, North Carolina, USA). Statistical significance was set at $P < 0.05$. For the outcomes defined below, two mixed regression model structures (Dohoo et al., 2009) with random effects at the cow, day , and preservation levels were built including any two- and three-way interaction terms of predictors as explained below. Univariable models were assessed, and any predictors with $P < 0.20$ were further analyzed in multivariable models. Back-transformed marginal predictions and pairwise comparisons, as well as contrasts with Bonferroni corrections of *P*-values for multiple comparisons, were also analyzed for each model where indicated.

The first model used a log transformed S/P ratio as the outcome for IFN-γ production analysis. The model included variables for transport media (dichotomous), treatment (five categories), and day (three categories). Treatment categories included PBS (base value), PWM, ConA, ConA + IL-12 p40, and IL-12 p40. Day categories were labelled as day 1, 5, and 8 to represent the days when cells were harvested after stimulation.

The second model used percent of mononuclear cell viability as the outcome for cell viability analysis. This percent was defined as the percent of total cells counted by flow cytometry that were viable mononuclear cells. Total cells counted (equivalent to 100%) included dead and viable mononuclear cells, as well as dead and viable polymorphonuclear cells. No transformation was required for this outcome in the

regression analysis. The same predictors were included as described for the first model. No other cow- or herd-level factor information was collected at the time of sampling, and therefore, no other predictors were available to be included in the models.

6.4 Results

6.4.1 Descriptive Data

There were a total of 300 observations, corresponding to whole blood samples from the ten cows. Five treatments were performed on each of unpreserved and preserved samples, on each of three time periods. There were 14 missing S/P ratio results and two missing flow cytometry results due to hemolyzed samples (no pellet-serum delineation) or due to ELISA OD results that exceeded the recordable limit.

6.4.2 Stimulation Effect and IFN-γ Production

For the 286 observations from the ten healthy cows, untransformed S/P ratios ranged from -9.8% to 468.1%, with a mean of 20.4% and a median of 1.3%. This highly right skewed outcome was therefore adjusted to be greater than zero by adding ten units to every value followed by a log transformation for regression analysis.

6.4.2.1 Multivariable Analysis

Overall, in pairwise comparison analyses, PWM and ConA stimulation effect was significantly different from no stimulation (PBS control) ($P < 0.01$), but IL-12 p40

alone was never significantly different from PBS. Furthermore, there was an overall significant effect of time (day) ($P < 0.01$). The three-way interaction among time (day), stimulation method, and use of transport medium was significant ($P < 0.01$) (Table 6.1). Pokeweed had a strong stimulating effect (Figure 6.1), particularly on day 1, but this effect in unpreserved samples was calculated with only six samples as the other four samples were over-stimulated beyond the maximum detection abilities of the ELISA. In addition, among preserved samples alone, the use of any one of the three stimulants was significantly different ($P < 0.01$) from the PBS control on both days 1 and 5. Although ConA and ConA + Il-12 p40 produced very similar results (Figure 6.2), only stimulation with ConA alone in preserved samples on day 5 continued to produce IFN-γ above the positive cut-point (S/P = 15%) for the ELISA kit.

Table 6.2 depicts the difference in S/P ratios for each stimulation treatment as compared to the control (PBS) over time (days) for unpreserved samples and samples preserved with transport media, by showing the proportion of the contrast outcome (difference) in S/P ratios within each of the three stimulation methods on day 5 or day 8, as compared to the results on day 1. Numerically, IFN-γ production was generally higher on days 5 and 8 in preserved samples as compared to unpreserved samples. However, using Bonferroni corrections, only the combination of ConA + IL-12 p40 showed statistically significant stimulation of IFN-γ production on day 8 in preserved samples as compared to unpreserved samples ($P < 0.05$).

6.4.3 Mononuclear Cell Viability

For the 298 observations from ten healthy cows, percent of viable mononuclear cells ranged from 7.7% to 81.8%, with a mean of 50.2% and median of 54.9%. No transformation was required for regression analysis.

6.4.3.1 Univariable Analysis

Univariable regression models showed highly significant day effect ($P < 0.01$) and transport medium effect ($P < 0.01$) on the percentage of live mononuclear cells counted by flow cytometry. Stimulant use, however, had no significant effect, and was subsequently not included in the multivariable model, after checking for confounding effects and model testing with and without this parameter.

6.4.3.2 Multivariable Analysis

The final mixed linear multivariable regression model showed a highly significant interaction ($P < 0.01$) between day and transport medium parameters. When comparing preserved and unpreserved samples (Figure 6.3), the proportion of mean live mononuclear cells present in unpreserved samples on day 8 was 45.3% of the mean amount present on day 1. However, in samples preserved with the transport media, 76.4% of those present on day 1 were still viable on day 8.

The mean mononuclear cell viability was 7.9% higher (95% CI: -5.9% to 21.7%) ($P = 0.04$) for preserved samples versus unpreserved samples on day 5 (as compared to day 1). This difference was 16.6% (95% CI: 2.8% to 30.5%) on day 8 (as compared to day 1) in favor of preserved samples ($P < 0.02$). Within day 8 there was also significantly more viable cells in preserved samples ($P < 0.01$).

6.5 Discussion

The practical application of a cell viability transport medium would allow for more efficient utilization of blood diagnostic tests, such as the cell-mediated IFN-γ assay, in veterinary medicine applications. In particular, the use of such a transport medium would be highly beneficial to an increased application of the IFN-γ assay as part of Johne's disease control programs. This study assessed the use of the SCSR-T™ transport media in bovine whole blood samples. For this assessment, an evaluation was performed using nonspecific mitogens, prior to evaluating the effects with specific MAP antigens. Overall, our results point to a benefit for incorporating a cell transport medium in whole blood samples to allow for longer travel times from on-farm collection to processing in an appropriate laboratory.

6.5.1 Stimulation Effects and IFN-γ Production:

As one method for detecting cell viability over time, stimulation of IFN-γ production assessment indicated better long-term results with preserved blood samples. Nonspecific stimulation was noted with both PWM and ConA, indicating that viable B and T cells were present over time (Table 6.2). The benefit of the transport media for IFN-γ production in our study was observed with ConA and ConA + IL-12 p40 stimulation on day 5 post-collection.

As nonspecific mitogens can be used for positive controls in the IFN-γ assay, a positive response to the mitogens indicates that viable and healthy immune cells are present in the sample at the time of testing (Stabel and Whitlock, 2001). By extending cell life, the actions of these nonspecific mitogens can also be more accurately assessed over time, thereby aiding their use as appropriate reference standards against which the actions of specific antigens could be evaluated over time.

In our study, PWM overstimulated IFN-γ expression on day 1, particularly for unpreserved samples. This overstimulation with PWM has been documented in the literature (Plain et al., 2012; Stabel, 1996), where some of this effect may be due to the action of PWM on primarily B cells in addition to T cells (Gershwin et al., 2005, p. 105; Stabel, 1996), indicating in our study a greater presence of B cells on day 1. In contrast, ConA acts solely on T cells (Gershwin et al., 2005, p. 105; Stabel, 1996). Therefore, Stabel (1996) suggests that ConA would be a better stimulation agent in cell-mediated assays for samples from preclinical Johne's diseased animals. Stabel (1996) had also observed an increased IFN-γ production by non-stimulated WBCs after two incubation days, suggesting that this occurrence could stem from a possible spontaneous secretion of some T cell activating factors from the mononuclear cells *in vitro*.

It was difficult to accurately compare the results of our study to others analyzing the effects of mitogens, as there are no standard concentration and incubation methods throughout the literature. However, in an effort to maintain some similarity, we used the more frequently reported dosage of 10 μg/ml for each of the mitogens (Robbe-Austerman et al., 2006; Stabel, 1996), even though previous work in our laboratory showed that a lower dosage of 5 μg/ml may also be generally effective (data not shown).

In contrast, a previous veterinary clinical immunology study suggested an optimal concentration of 15 μg/ml for ConA and only 5 μg/ml for PWM (Barta and Oyekan, 1981).

Furthermore, many of the studies found in the literature analyzing IFN-γ production either use heparinized blood or do not report the use of heparinized tubes during blood collection. Again, to maintain some similarity, we chose to use heparinized vacutainer tubes for blood collection. However, it has been previously noted that heparin can have a lymphocytolytic effect due to a subsequent increase of free fatty acid concentration in heparinized plasma (Klein et al., 1991). This effect may be inconsequential if lymphocytes are isolated within 48 hours of blood sample collection (Klein et al., 1991). Despite this potential negative effect of heparin, our cell populations still showed healthy activity and viability during our study period, particularly for blood preserved with the transport medium.

Although there is little information in the literature, the pro-inflammatory cytokine IL-12 seems to act as an inductor, polarizing Th1 immune responses (Stabel, 1996; Stabel, 2010). Specifically, IL-12 synergistically with IL-18 induces cell-mediated immunity against mycobacteria by promoting the release of IFN-γ through the activation of Th1 cells and NK cells (Price et al., 2006; Stabel, 2010). When IL-12 and IFN-γ production was negatively regulated by the increased presence of anti-inflammatory cytokines, such as IL-10, a Th2 immune response can develop (Stabel, 2010).

Jungersen et al. (2005) recommends the addition of IL-12 within 20 hours of blood collection in order to aid in bolstering a weaker WBC response. In our study,

IL-12 p40 alone was unable to elicit detectable IFN-γ production above the cut-off for a positive result, but it was able to augment the effect of ConA. It would, therefore, be recommended to further evaluate this effect over time with a larger sample size, as very little information is available in the Johne's disease literature aside from IL-12's co-stimulatory effect with specific mitogens (Mikkelsen et al., 2009).

6.5.2 Mononuclear Cell Viability

The results of our study indicated an advantage to incorporating a transport media at time of collection, as viable mononuclear cells were still present at eight days post-collection as determined with flow cytometry (Figure 6.3).

Knowledge of mononuclear cell viability over time in both preserved and unpreserved whole blood samples can enhance our understanding of the results expected with IFN-γ assays post-blood collection over time. A simple method to assess viability *in vitro* involves the utilization of propidium iodide dye (Davey and Kell, 1996). In our study, we had lysed the RBCs and used gating and propidium iodide dye in the flow cytometric analysis to separate viable and non-viable mononuclear and polymorphonuclear cells. We maintained a standard of 10,000 events each time in order to accurately compare percent of viable mononuclear cells among the three time points. Other options for cell marking for use with flow cytometry include trypan blue dye (Kristensen et al., 1982) or specific immune markers for T cell subpopulations with Ficoll fractionation (Kristensen et al., 1982; Price et al., 2006).

Furthermore, several cow-level, environmental, and laboratory factors can lead to variation in assay results within individual cows. In particular, cow-level factors

include age, stress, health conditions, nutrition, and pregnancy. In addition, laboratory factors can make comparison between studies difficult. Some of these factors include not only methodology and mitogen concentrations, but also incubation periods, temperature variations, type of culture media, fetal calf serum use, and presence of possible inhibitors (Kristensen et al., 1982).

6.5.3 Conclusion

Through examining the use of blood cell preservation with transport media in bovine whole blood samples, this study's objective was to analyze the cell preservation capabilities of the media with regards to lifespan of mononuclear cells and stimulation of IFN-γ non-specifically. Our results show that cell preservation with a transport medium allowed for extended cell viability up to eight days post-collection, with optimum cell preservation effects at five days post-collection. Therefore, a benefit to incorporating a cell transport medium in whole blood samples was observed, which would subsequently enhance the potential use of IFN-γ assays. The benefits are not exclusive to Johne's disease, but extend to any assay that requires whole blood, cell-based diagnostics.

6.6 References

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Table 6.1: Interferon gamma release analysis model estimates and *P*-values of the predictors for treatment (TX), preservation (TM), and day; the two- and three-way interactions; and the variances for all random effects on the log-transformed sample to positive ratio outcome.

Factor or Effect		Range of LSM[a] or Estimate		*P*-value
		TM = 0	TM = 1	
TX	PBS (control)	0[b]	0	<0.0001[c]
	Pokeweed	2.1519-5.6697	2.7792-4.6615	
	Concanavalin A	2.0956-3.5527	2.7380-3.6065	
	Concanavalin A + Interleukin-12	1.8612-3.5215	2.8275-3.6958	
	Interleukin-12	2.0731-2.3551	2.2055-2.3654	
TM				0.0385
Day				<0.0001
Interaction	tx*tm			0.0003
	tm*day			0.0099
	tx*day			<0.0001
	tx*tm*day			0.0048
Variance	Between cow	0.1157 (30.3%[d])		
	Between day	0.0151 (4.0%)		
	Between TM	0.0678 (17.8%)		
	Residual	0.1830 (47.9%)		

[a] Estimates for treatments presented as a range of least squares means (LSM) over time. Individual estimates for preservation and day are not presented, as both factors are involved in the interaction with treatments.
[b] ELISA results (n=60) for all the control treatments over time in both preserved and unpreserved samples were consistently zero and therefore not included in this model.
[c] *P*-value for complete treatment factor
[d] Proportion of total unexplained variance

Table 6.2: Change in sample to positive ratios of interferon gamma production by using a stimulant (pokeweed mitogen, PWM; concanavalin A, ConA; or interleukin-12 p40 potentiated ConA, ConA + IL-12 p40; or IL-12 p40 alone) versus no stimulation (phosphate buffered saline) over time (days), for white blood cells unpreserved or preserved with a transport medium (SCSR-T™; NonInvasive Technologies, Maryland) supplemented in whole blood samples from ten healthy cows.

	Blood Alone			Blood with Transport Medium		
	Day			Day		
	1	5	8	1	5	8
PWM	265.2[a] (6[c]) [98.4-432.2][b]	23.0[a] (9) [6.4-39.5] {8.7}[d]	-1.4 (9) [-7.1-4.4] {-0.5}	95.8[a] (10) [44.3-147.3]	19.9[a] (10) [5.4-34.4] {20.8}	6.1 (10) [-2.1-14.3] {6.4}
ConA	24.9 (10) [8.2-41.6] [a]	6.9 (9) [-2.0-15.8] {27.7}	-1.8 (9) [-7.4-3.7] {-7.3}	26.8[a] (10) [9.2-44.5]	15.9 [a] (10) [3.2-28.6] {59.3}	5.5 (10) [-2.5-13.4] {20.3}
ConA + IL-12 p40	23.8[a] (10) [7.6-40.1]	6.5 (9) [-2.2-15.2] {27.4}	-3.6 (8) [-8.9-1.6] {-15.3}	30.3[a] (10) [11.0-49.6]	13.5[a] (10) [1.9-25.2] {44.8}	6.9 (10) [-1.6-15.4] {22.8}
IL-12 p40	-2.0 (10) [-7.4-3.3]	-0.4 (9) [-6.4-5.5]	0.4 (8) [-6.1-6.9]	-0.9 (10) [-6.6-4.8]	0.1 (10) [-6.3-6.5]	0.6 (10) [-5.5-6.8]

[a] Significantly different ($P < 0.01$) sample to positive ratio compared to no stimulation (phosphate buffered saline) as determined by Bonferroni corrections of *P*-values for multiple comparisons.
[b] 95% confidence interval
[c] Total number of observations
[d] Proportion (%) of the contrast outcome (difference) in sample to positive ratio values by using a stimulant (pokeweed mitogen, PWM; concanavalin A, ConA; or interleukin-12 p40 potentiated ConA, ConA + IL-12 p40) over no stimulation (phosphate buffered saline) on day 5 or day 8 as compared to the results on day 1.

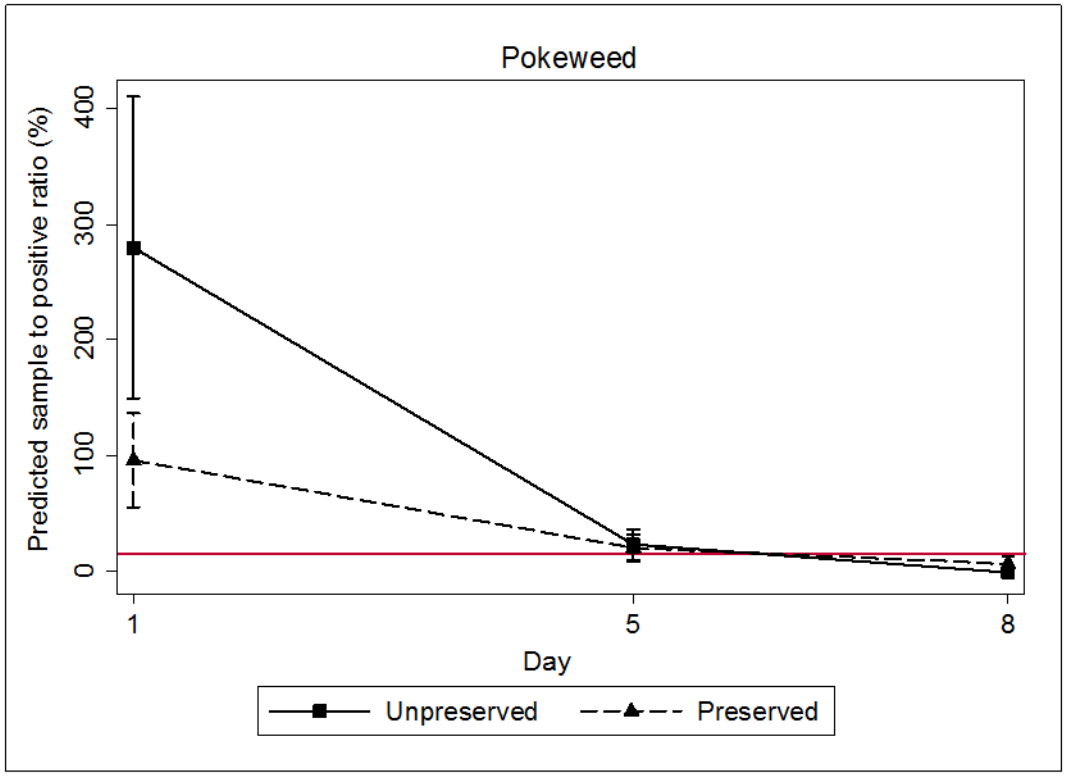

Figure 6.1: Predicted sample to positive ratios (with 95% confidence intervals) of interferon gamma production through stimulation with pokeweed mitogen over time (days), for whole blood samples, either unpreserved or preserved with a transport medium (SCSR-T, NonInvasive Technologies), that were collected from ten healthy cows.

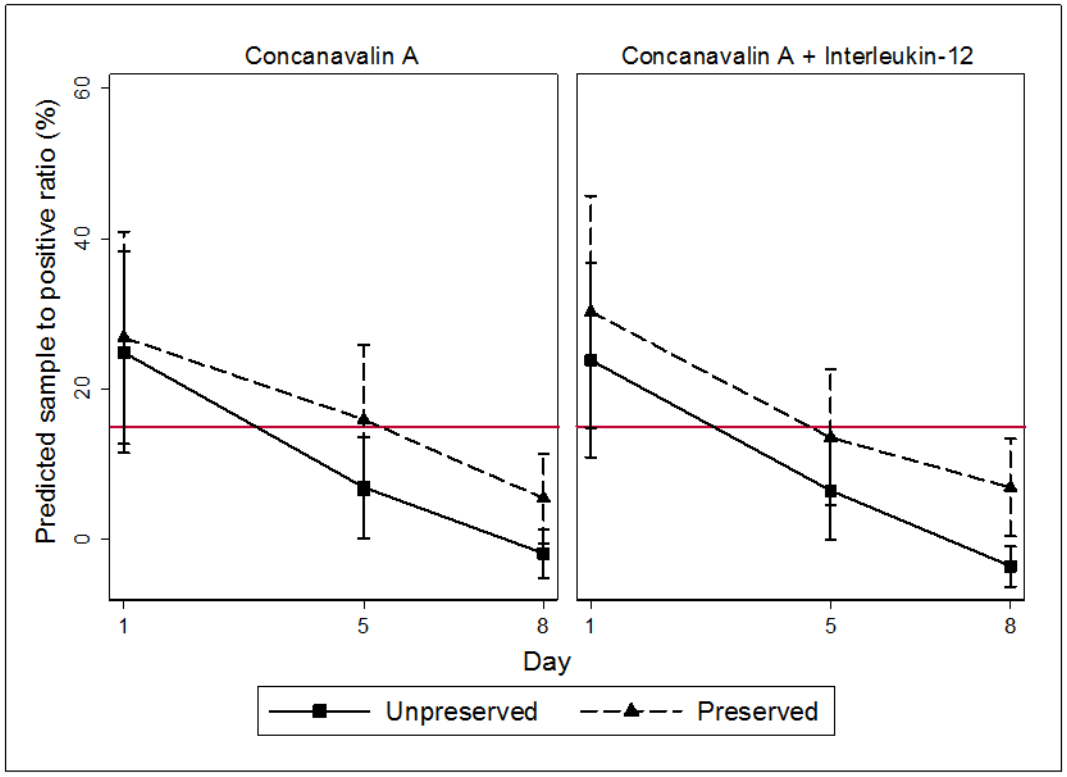

Figure 6.2: Predicted sample to positive ratios (with 95% confidence intervals) of interferon gamma production through stimulation with concanavalin A or interleukin-12 potentiated-concanavalin A over time (days), for whole blood samples, either unpreserved or preserved with a transport medium (SCSR-T, NonInvasive Technologies), that were collected from ten healthy cows.

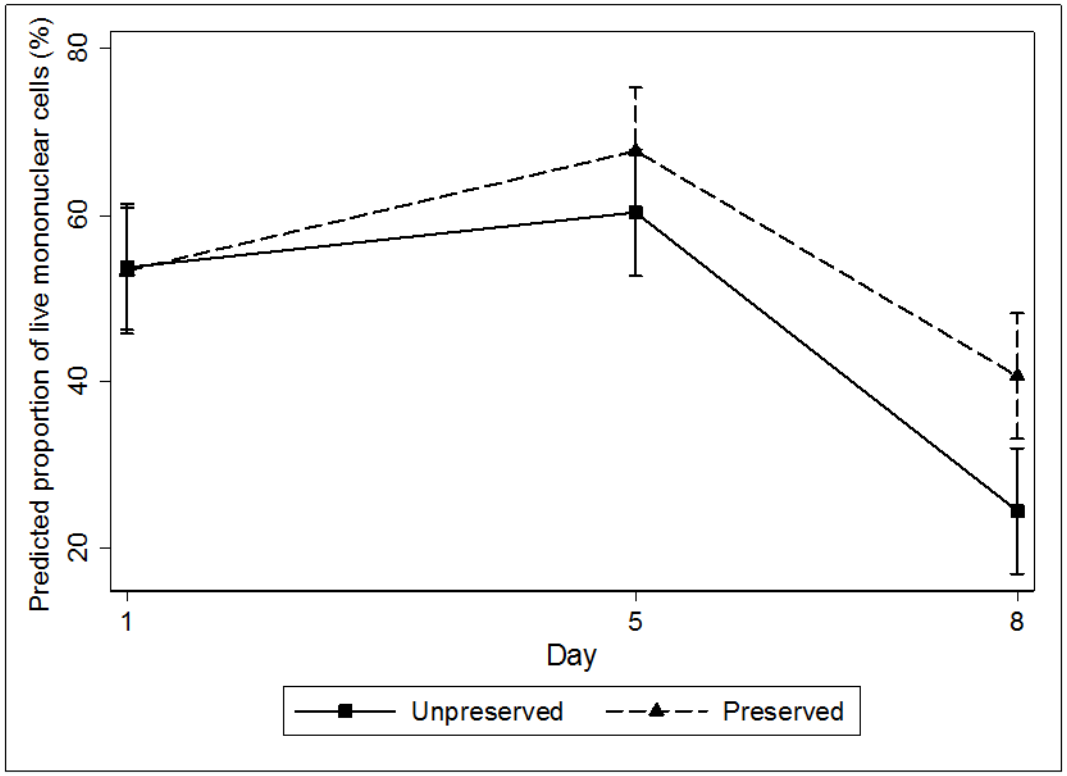

Figure 6.3: Predicted average proportion of mononuclear cell viability (with 95% confidence intervals) over time (days), for whole blood samples, either unpreserved or preserved with a transport medium (SCSR-T, NonInvasive Technologies), that were collected from ten healthy cows.

CHAPTER 7. SUMMARIZING CONCLUSIONS

Paratuberculosis, or Johne's disease, is a serious production-limiting disease of dairy cattle (Lombard et al., 2005) worldwide (Collins, 2003; Singh et al., 2013) that is difficult to diagnose due to the imperfect assays at our disposal (Nielsen and Toft, 2008; Whitlock, 2009). Because there is no treatment or vaccine available for dairy cows to cure or prevent infection with the causative organism *Mycobacterium avium* subsp. *paratuberculosis* (**MAP**) (Whitlock, 2010), adequate diagnostic tests become essential to implement the Johne's control and management programs necessary to minimize the effect of the disease within a herd (Whitlock, 2010; Garry, 2011). Furthermore, rising concerns about milk as a MAP transmission mode to humans, and subsequent hypothesized zoonotic risks for Crohn's disease (Herman-Taylor and Bull, 2002; Gill et al., 2011; Chiodini et al., 2012; Serraino et al., 2014), highlight the need for more effective diagnostic tests, particularly for milk samples. Unreliable test results, due to variation with the commonly used pathogen and antibody detection methods (culture, real-time polymerase chain reaction (**qPCR**), and enzyme linked immunosorbent assay (**ELISA**)) for paratuberculosis (Appendix A shows the monthly variation observed in our data), can lead to producer nonparticipation in Johne's control programs. Consequently, being able to identify patterns in MAP shedding and factors influencing MAP detection are important to implementing effective management and control programs in a cost- and time-efficient manner for producers.

The ability to detect MAP-shedding was a common facet to the majority of this research. The target condition for Chapters 2 to 4 was a MAP-infectious cow, or a cow with detectable shedding (Nielsen and Toft, 2008). Chapter 2 focused on fecal shedding of MAP, Chapter 3 on milk and colostrum shedding of MAP, and Chapter 4 on detecting antibodies to MAP in milk samples. For the remaining chapters, the focus shifted to developing novel methods of early MAP detection for the benefit of identifying MAP-infected and MAP-infectious cows as early as possible in order to gain the maximum benefit from control and management programs. Therefore, the individual cows identified to contribute to the sampling data for this analysis were purposively selected as known to be shedding MAP in their feces, as consistently test-negative on all previous testing from test-positive herds, or as healthy from test-negative herds.

7.1 Patterns in Detecting MAP Shedding: Feces

The focus for Chapter 2 was to assess the detection ability of three commonly used fecal pathogen detection methods (solid culture, broth culture, and qPCR) and determine the effect of time (season and lactation stage) on detectable MAP shedding over the course of one year of monthly sampling from 51 MAP-infectious cows. Of the three assays evaluated, qPCR had superior sensitivity (**Se**), particularly in the dry and post-partum periods, although sample numbers were low for these periods. This contrasted with previous reports of lower qPCR Se from a study utilizing the same qPCR kit as employed in our research (Alinovi et al., 2009). An advantage of using

qPCR, particularly in known low-prevalence herds, is that the genetic detection of MAP can occur from both viable and nonviable bacteria and happens in the absence of decontamination steps that could further decrease low numbers of viable bacteria within a sample. In comparison, culture methods are dependent on the presence of live bacterial cells within the sample, and enumeration of viable cells is negatively affected by decontamination protocols and the presence of faster-growing competitive organisms (Bölske and Herthnek, 2010).

Although statistically significant associations between season, lactation stage and MAP recovery were not identified when accounting for multiple comparisons due to low numbers of samples in some categories, apparent patterns were observed for qPCR results. Additionally there was a seasonal pattern related to failure of solid culture decontamination methods. In general, seasons with more similar climatic temperatures were also more similar in qPCR detection ability, with improved Se in colder months. Although we did not find an association between season and culture detection of MAP, the association between season and qPCR MAP detection agree with other studies that looked at the effect of season on MAP growth in culture (Crossley et al., 2005; Norton et al., 2010).

As a benefit to control programs, the risk of environmental contamination and subsequent infection of calves could be indirectly calculated using qPCR cycle threshold (**Ct**) values (using the same kit) as an indicator of the degree of fecal MAP shedding in dams without simultaneous culture testing. Our results indicated that positive shedding (culture reference standard) corresponded to <35.0 Ct and heavy shedding to <29.0 Ct, which was similar to the results obtained by Leite et al. (2013), using the same kit.

7.1.1 Conclusions

The results of Chapter 2 support the use of direct fecal qPCR as part of a Johne's herd management program, particularly for known infected herds. This chapter also highlighted benefits to using qPCR in months where culture contamination is more likely. Consideration of Ct values further extends the application of qPCR within MAP control programs, as lower Ct values are indicative of greater MAP bacterial load in a cow's feces. This allows for prioritized management of high shedding cows, and could ultimately increase the success of herd MAP control programs.

7.2 Patterns in Detecting MAP Shedding: Milk and Colostrum

The focus of Chapter 3 was to assess the detection of MAP in milk and colostrum samples using the same three assays (solid culture, broth culture, and qPCR) and to compare with results obtained from fecal samples. Any effects of season and lactation stage that could benefit improved monitoring of infection risk to calves and improved detection of MAP within milk samples for public health concerns were considered. Overall, regardless of concurrency of testing, results indicated that it was approximately four times more likely to detect MAP within feces than milk from the cows, when using the same molecular detection method, while just under half of the MAP detected in feces was also detected in colostrum, using qPCR. For MAP-infectious

cows, qPCR had the best detection ability, particularly for milk samples collected during summer in Atlantic Canada.

It is possible that low numbers of bacteria in the milk or colostrum samples, or loss of viable cells via decontamination techniques, may have led to no growth on culture media and, thereby, false negatives for low-shedding animals that could still be detected by qPCR. Traditionally, MAP growth on cultures of milk and colostrum samples is difficult for a variety of reasons, but due mainly to an inherently lower MAP concentration in these samples (Gao et al., 2005; Pinedo et al., 2008). In addition, the disease stage of cows within study groups could affect the degree of bacterial shedding into milk or colostrum (Bradner et al., 2013). Therefore, there is a lot of variation in the literature regarding Se of pathogen detection methods for paratuberculosis in these samples (Slana et al., 2008; Gao et al., 2009; Bradner et al., 2012; Bradner et al., 2013). Our results were within the bounds of those reported by others, perhaps due to our choice of target condition. Furthermore, shedding in milk and feces may not consistently coincide (Gao et al., 2009), as seen in our analyses (see also Appendix A) with approximately one-third of the MAP detected in feces concurrently detected in milk, regardless of testing method. More specifically, when solely using qPCR testing, MAP was approximately five times more likely to be identified in feces than in milk.

7.2.1 Conclusions

The results of Chapter 3 identify qPCR as an improved MAP identification test, as compared to traditional culture methods, for milk and colostrum. This chapter also highlighted a seasonal pattern for increased qPCR detection of MAP in milk samples

during summer, and an improved harmony between milk and fecal shedding in summer. Understanding patterns of detectable MAP shedding in milk and colostrum can reveal more efficient detection strategies for these samples.

7.3 Patterns in Detecting MAP Antibodies: Milk ELISA

The focus of Chapter 4 was to assess the variation in MAP antibody detection using a commercial ELISA kit for milk samples over time (season and lactation stage), and compare results with fecal test results in MAP-infectious cows. The analysis performed in lower MAP prevalence herds (3% to 15%) supports reports of lower milk ELISA Se than standard fecal diagnostics, with a milk Se of approximately 30% and specificity (**Sp**) of 99%. In comparison, Slana et al. (2008) also found higher Sp along with Se ranging between 21% and 67%, using the same commercial ELISA kit on individual cow milk samples. Comparisons among milk or even serum ELISA studies for Johne's disease can be difficult due to the range of study types, case and target definitions, and kit and methodology employed. For example, McKenna et al. (2005) observed that serum ELISA Se was approximately twice as high if estimated against a reference standard of positive fecal culture, versus tissue culture. As expected, our data analyses revealed improved milk ELISA detection ability in MAP-infectious cows with increased MAP shedding.

Generally, ELISA Se should increase with each stage of disease, with increasing numbers of shed bacteria (Carpenter et al., 2004; Clark et al., 2008), and with increasing

age or parity (Toft et al., 2005). This chapter also highlighted higher milk ELISA numeric results in later lactation, in contrast to a study by Nielsen et al. (2002) that found increased ELISA Se at the beginning of lactation. Milk antibody concentration may be greater both in early (first and second weeks) and late (greater than 45 weeks) lactation than in the third to twelfth weeks of lactation (Nielsen et al., 2002; Lombard et al., 2006) due to colostral antibody levels, milk dilution effects, and Johne's disease stage (Nielsen and Toft, 2012). In addition, our analyses found that the effect of lactation was affected by season, with higher results during winter months. However, the variation in scores was sometimes numerically small and did not necessarily cross the positive threshold.

7.3.1 Conclusions

The results of Chapter 4 indicate overall milk ELISA Se of 30% and Sp of 99.3%, with increasing ELISA Se in relation to increased fecal shedding, age, or parity. In addition, there was a significant effect for season (winter versus summer) and lactation stage (increasing days in milk) on ELISA continuous results that more clearly explains the variation over time. Again, accurate knowledge of shedding and diagnostic patterns is vital for reducing MAP transmission risks and for development of improved diagnostic and screening protocols.

7.4 Novel Early Detection: MAP Specific Proteins

The focus of Chapter 5 was two-fold. The first focus was to determine if a novel ELISA method, using an antigen coating incorporating early-secreted MAP protein tyrosine phosphatase A (**PtpA**) or protein kinase G (**PknG**) and lipase-like proteins, could be used as an early ELISA detection method in bovine milk, colostrum, or serum samples. The earlier the infection can be diagnosed, the sooner specific, efficient control mechanisms can be instigated (Bannantine et al., 2004; Stabel et al., 2007). With the novel protein acting as antigen, the novel ELISA could potentially measure antibodies specific to these proteins secreted at early-stage MAP infection for subclinical detection. However, in many of our runs, too much variation in negative controls made for too much uncertainty in the validity of sample results for diagnostic purposes. A large portion of Chapter 5 included looking at different concentrations of antigen-protein coating, different dilution ratios, and different negative controls, as well as comparison to samples from a MAP test-negative herd. One of the primary difficulties encountered was the very small and slow yield from protein overexpression attempts, making repeated attempts of the ELISA within a short period of time and for a large number of samples quite difficult. There was promise for the use of PknG and lipase-like proteins, but further research is required.

The second focus for Chapter 5 was evaluating the use of these two proteins as MAP-specific alternatives to the commonly used, protein-purified derivative Johnin as a stimulation antigen in the early-use interferon gamma (**IFN-γ**) assay. Detection of IFN-γ, although not necessarily indicating infection, identifies exposure and the potential for transmission in the herd (Zervens et al., 2013). Due to a limited time and budget for this portion of the research, only a very small sample size and few repetitions

were available for this pilot run. Unfortunately, a lot of variation was observed in our results. Nevertheless, the strong response to interleukin (**IL**)-12 p40 potentiated concanavalin A (**ConA**) stimulation indicated that viable lymphocytes were present in our samples and that there were no false-negative reactors present (Stabel and Whitlock, 2001). Interferon gamma was detected for both virulence proteins, especially when using IL-12 p40 potentiated PknG and PtpA.

7.4.1 Conclusions

Although the results presented in Chapter 5 are preliminary and not yet applicable for diagnostic test development, they indicate that PtpA or PknG and lipase-like proteins, as early MAP virulence proteins, are detected as MAP antigens in milk, colostrum, and serum samples of infected cows by both ELISA and IFN-γ assays. The identification of highly specific antigens for MAP for either improved IFN-γ assay or an early-use ELISA are imperative for developing an early MAP infection identification test or a highly reliable early-use, decision-support assay for MAP control, in calves or subclinical cows.

7.5 Novel Early Detection: Cell Viability

The focus of Chapter 6 was to assess the novel application of a human cell transport media in bovine whole blood samples to allow for longer sample transport times and, thereby, more efficient and practical utilization of blood diagnostic tests, such

as the early cell-mediated IFN-γ assay. Results indicated that cell preservation with a transport medium significantly increased mononuclear cell viability and IFN-γ response to IL-12 p40 potentiated ConA stimulation up to eight days post collection, with maximum benefit of live and healthy cells on day five, post collection.

An alternate method of assessing cell viability in preserved whole blood samples was to assess mononuclear cell viability with flow cytometry, as these cells are involved in IFN-γ production in cell-mediated immunity (Kristensen et al., 1982). Furthermore, dead cells were identified separately from live cells with the addition of propidium iodide dye.

7.5.1 Conclusions

The results of Chapter 6 revealed that cell preservation with the transport medium allowed for extended cell viability up to eight days post collection, with best results at five days post collection. This novel method can benefit the use of diagnostic assays requiring fresh whole blood samples, such as the early IFN-γ assay for paratuberculosis, by allowing for longer sample transit times.

7.6 Future Research Directions

The shedding patterns, novel early-secreted MAP protein use, and cell viability aspects of this research all provide new information for understanding and utilizing common, cow-level diagnostic strategies for paratuberculosis management. Every new

piece of information gleaned in paratuberculosis research adds another piece to the puzzle of how this complex and oft-times frustrating disease can be managed, to decrease the serious effects it has within the dairy industry. However, every new piece of information also uncovers more questions for further research directions.

Chapters 2 to 4 identified patterns of detecting MAP shedding over time, whether over season or over lactation. It would be of benefit to assess these patterns over a several year period, with a much larger sample size in cows of various MAP stages and herds of different MAP prevalence. In addition to this, other important factors to analyze include the impact of herd management, housing, and dietary changes on MAP fecal shedding, especially for cows within the dry and postpartum periods of lactation that are at greater risk of transmitting MAP to the highly susceptible calves. It has been suggested that increased stress levels, such as those produced by crowding, increased milk production and calving, changes in feeding practices and herd management, changes in environment (pasture), adverse weather conditions, and poor body condition, may also lead to seasonal trends (Jørgensen, 1977; McKenna et al., 2004; Crossley et al., 2005). Further investigation into the seasonal patterns of culture decontamination failures would also be of benefit. Literature suggests that culture decontamination failures may be due to diet and farm location, and are, therefore, likely to be affected by clustering (Whitlock et al., 1989; Whittington, 2009). Little is known about such possible causal factors for shedding and decontamination failure patterns, particularly for milk. However, this knowledge is important to enhance specific herd Johne's disease control management and diagnostic protocols, and warrants further research.

Unfortunately, current diagnostic tests are not reliable for early diagnosis of subclinical cows (Kalis et al., 2003). Therefore, the use of early MAP proteins, such as PknG and PtpA, as specific antigens and proteins in diagnostic tests, such as ELISA and IFN-γ, may be a step towards developing a rapid, yet sensitive and specific, early MAP identification tool. Further work in analyzing these virulence proteins in not only MAP-infectious cows but also in suspected exposed cows and calves from test-positive herds in comparison to test-negative herds, is required. Further research goals involve improvements to ELISA coating applications, including protein concentration and combinations, to improve sensitivity and specificity of the assay, as well as improvements to the IFN-γ assay using larger sample sizes and samples from heifers and calves.

Since cell-mediated responses are strong during early infection, IFN-γ testing can be used as a support mechanism to identify gaps in paratuberculosis management protocols that permit MAP exposure, especially for subclinical cows and calves (Collins, 1996; Kalis et al., 2003). Two preliminary methods evaluated in Chapter 6 to assess white blood cell viability over time to benefit IFN-γ testing included the nonspecific stimulation of IFN-γ and propidium iodide flow cytometric analysis. Further evaluation of these methods with MAP-specific antigens, as well as the ability of IL-12 to augment ConA stimulation ability, would be beneficial. Jungersen et al. (2005) recommends the addition of IL-12 within 20 hours of blood collection in order to aid in bolstering a weaker white blood cell response, and cautions that additional IFN-γ production by natural killer cells (Olsen et al., 2005) in younger animals could potentially elicit false positive results. It would be useful to further evaluate this effect over time with a larger

sample size to increase statistical power and decrease variability, as very little information is available in the Johne's literature, aside from IL-12's co-stimulatory effect with specific mitogens (Price et al., 2007; Mikkelsen et al., 2009; Plain et al., 2012). Larger sample sizes can also increase statistical power in evaluating causes of variability in results obtained. Some factors which can result in variability in mononuclear cell viability and stimulation assay results among individual cows include cow-level, environmental, and laboratory conditions (Kristensen et al., 1982). This knowledge can further improve the efficient use of this assay for paratuberculosis management and control programs.

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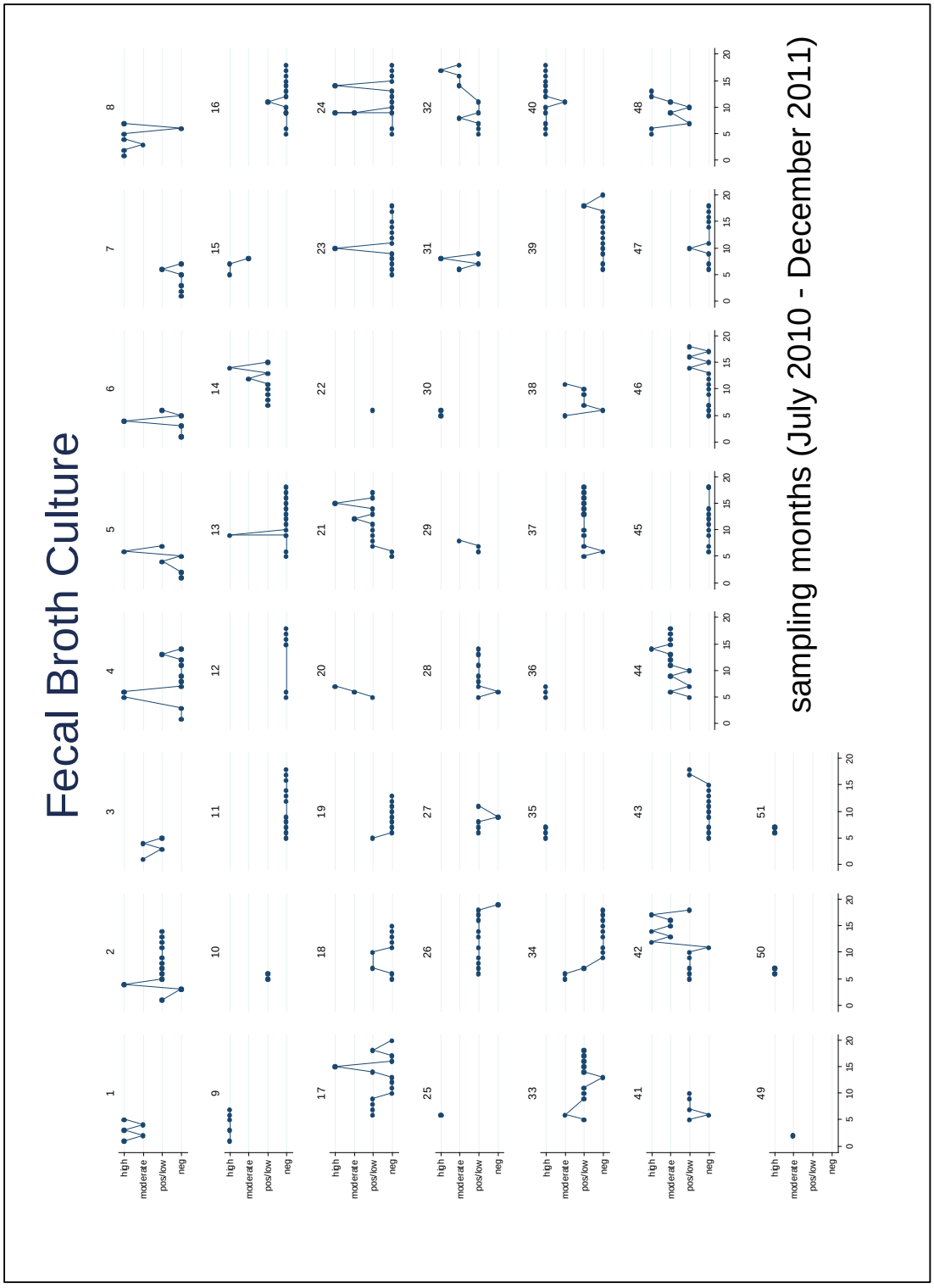
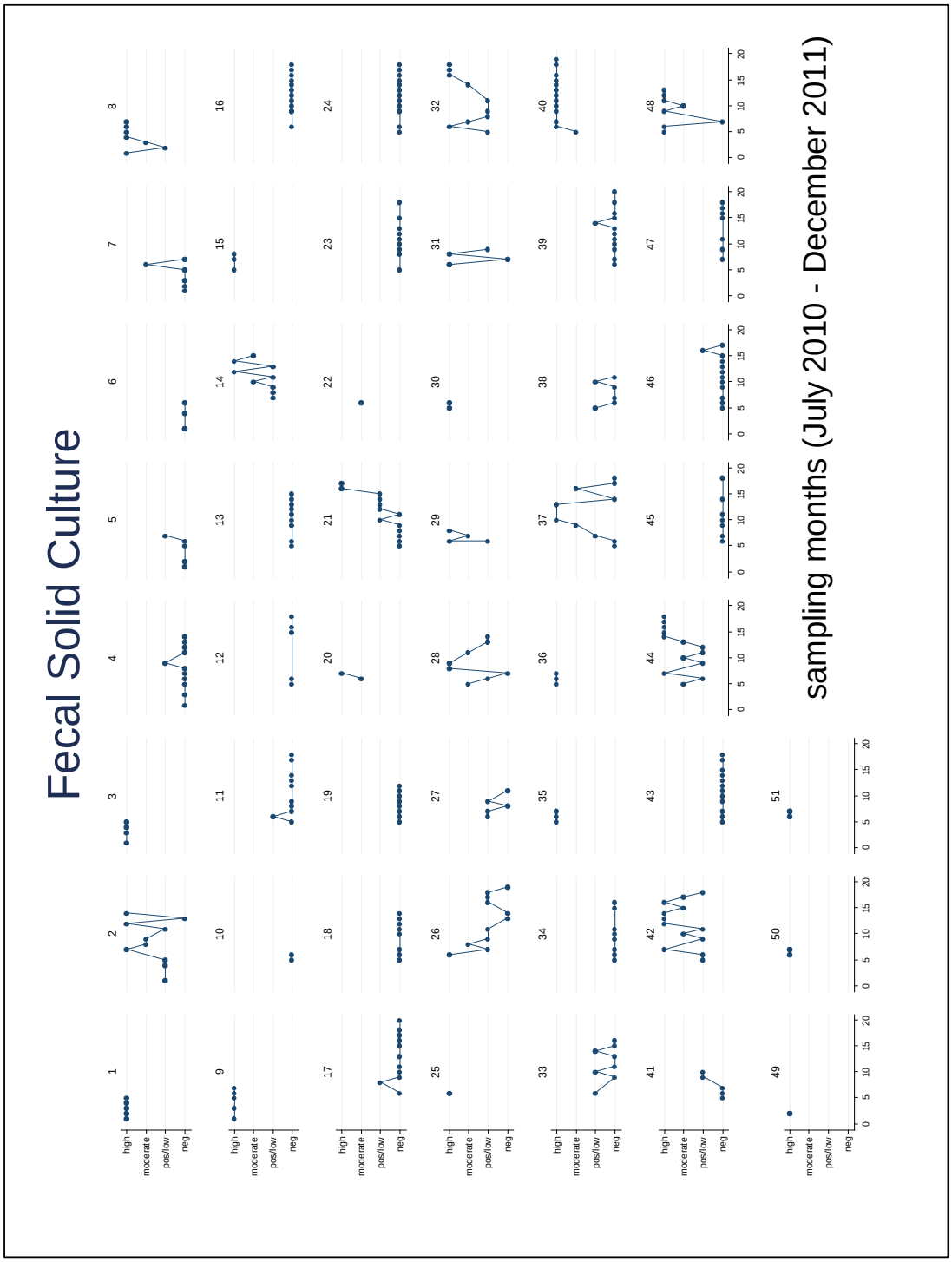
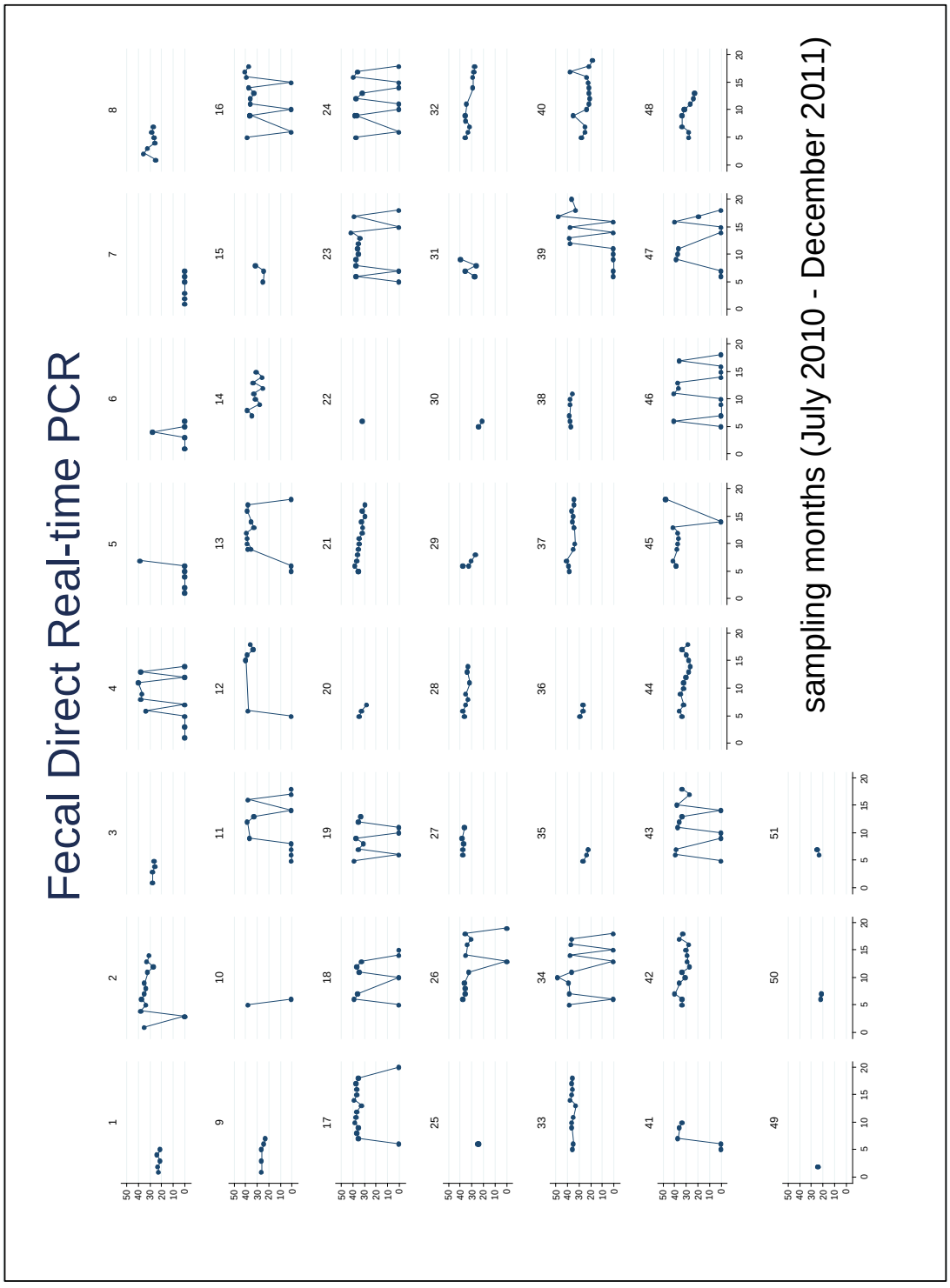
APPENDIX A

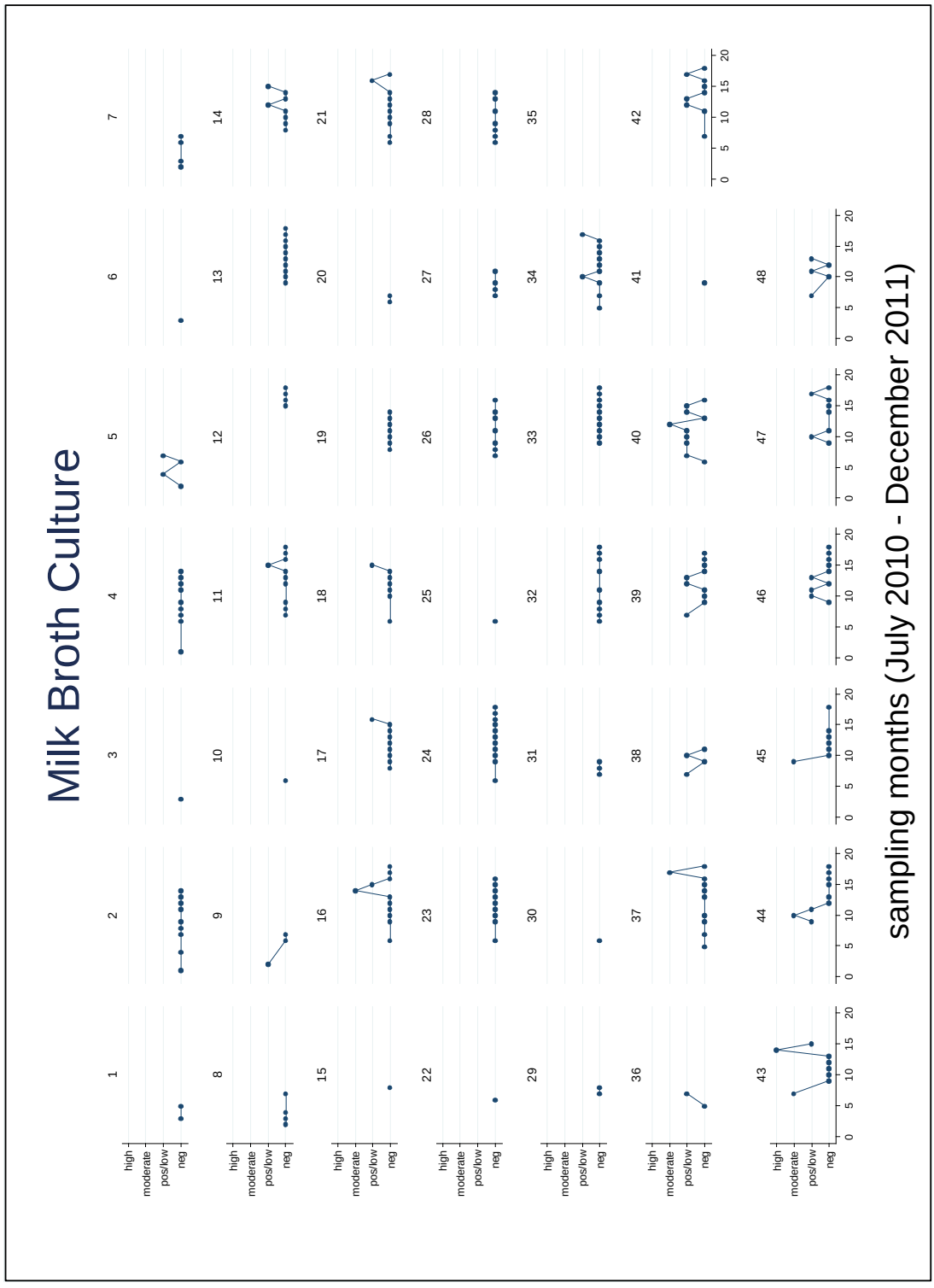
MONTHLY VARIATION OBSERVED IN FECAL, MILK, AND COLOSTRUM SAMPLES USING BROTH AND SOLID CULTURE, REAL-TIME PCR, AND ELISA ASSAYS

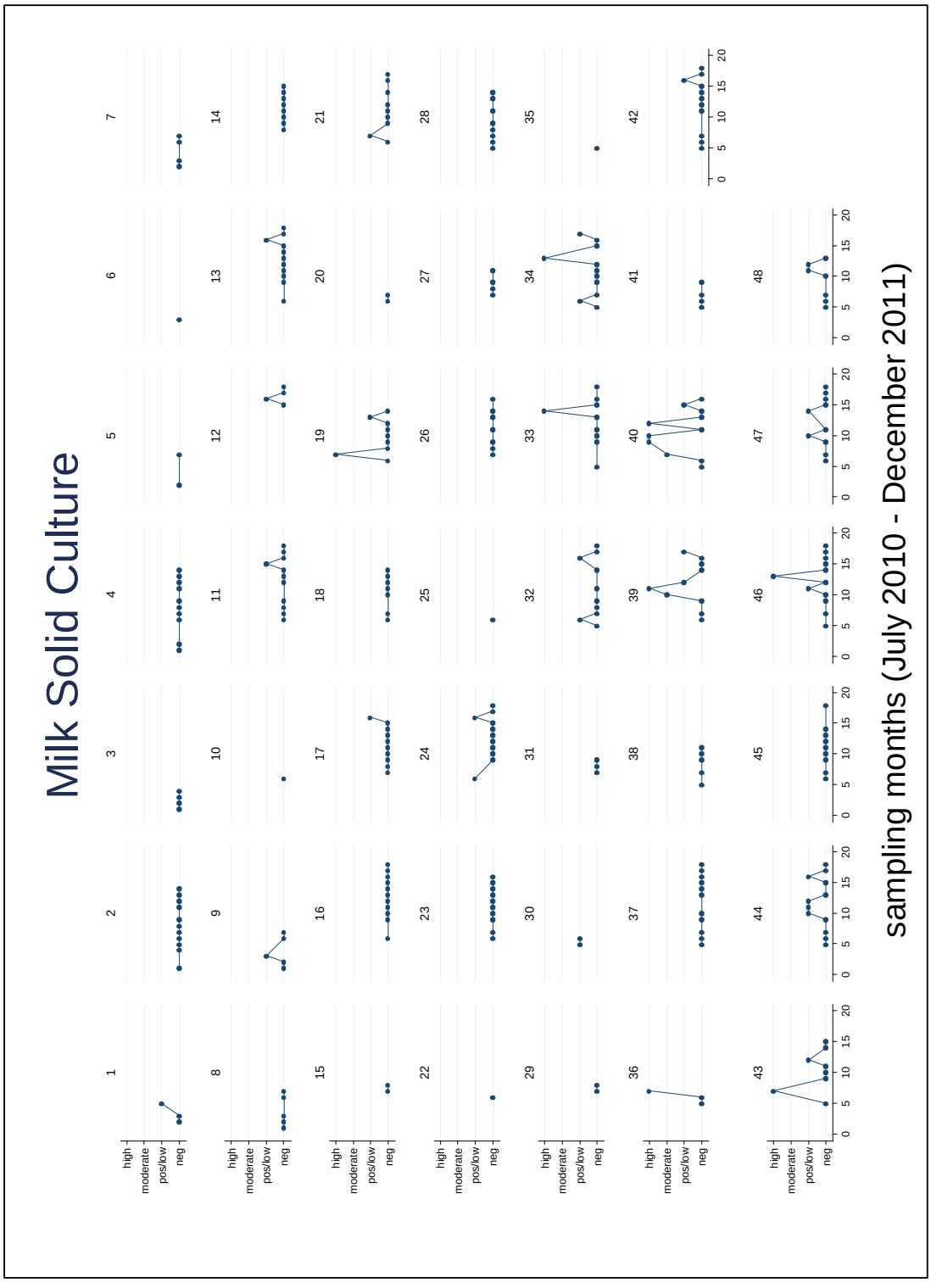

Fecal Broth Culture
sampling months (July 2010 - December 2011)
high
moderate
pos/low
neg

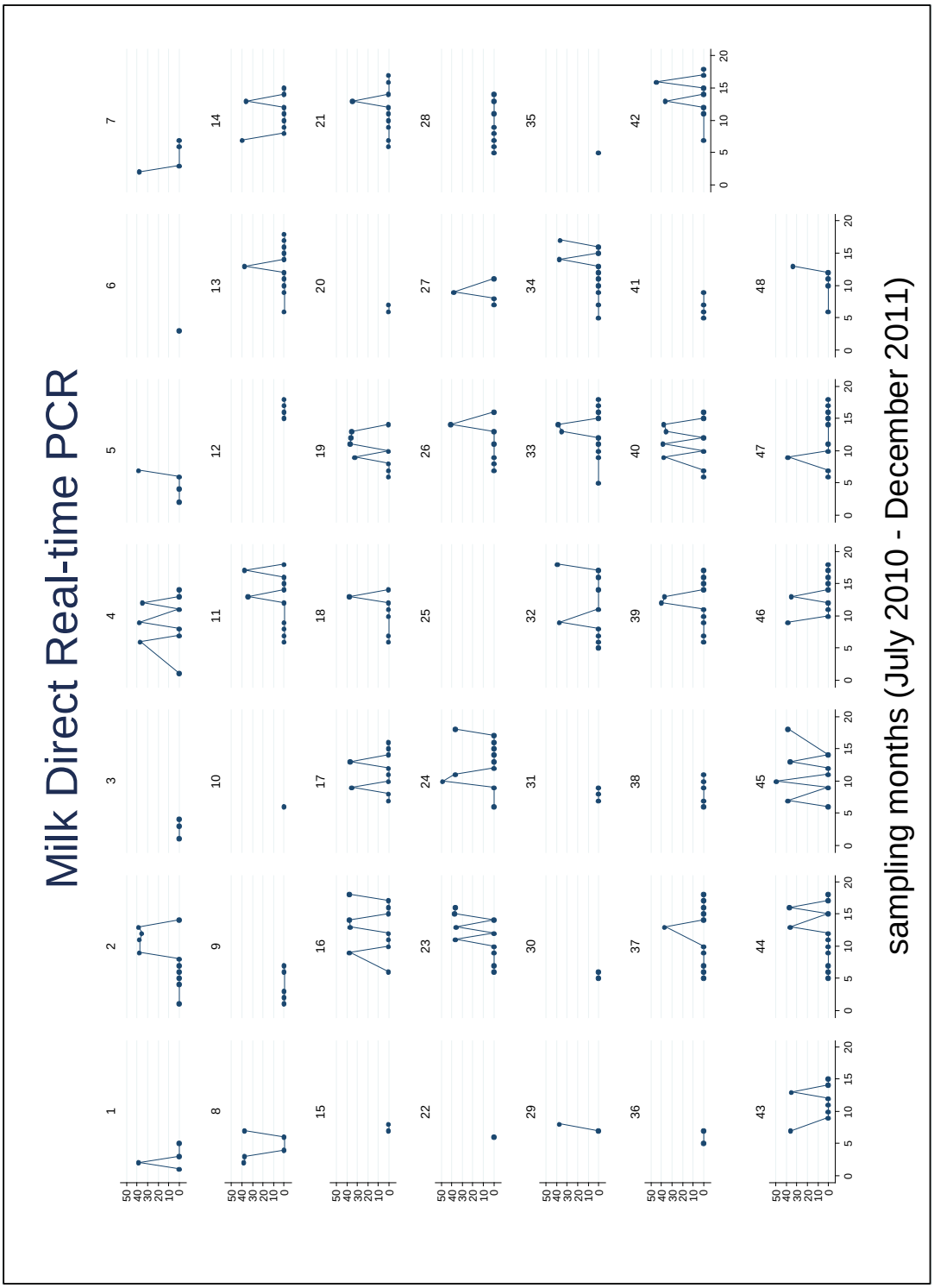

Fecal Solid Culture
sampling months (July 2010 - December 2011)
high
moderate
pos/low
neg

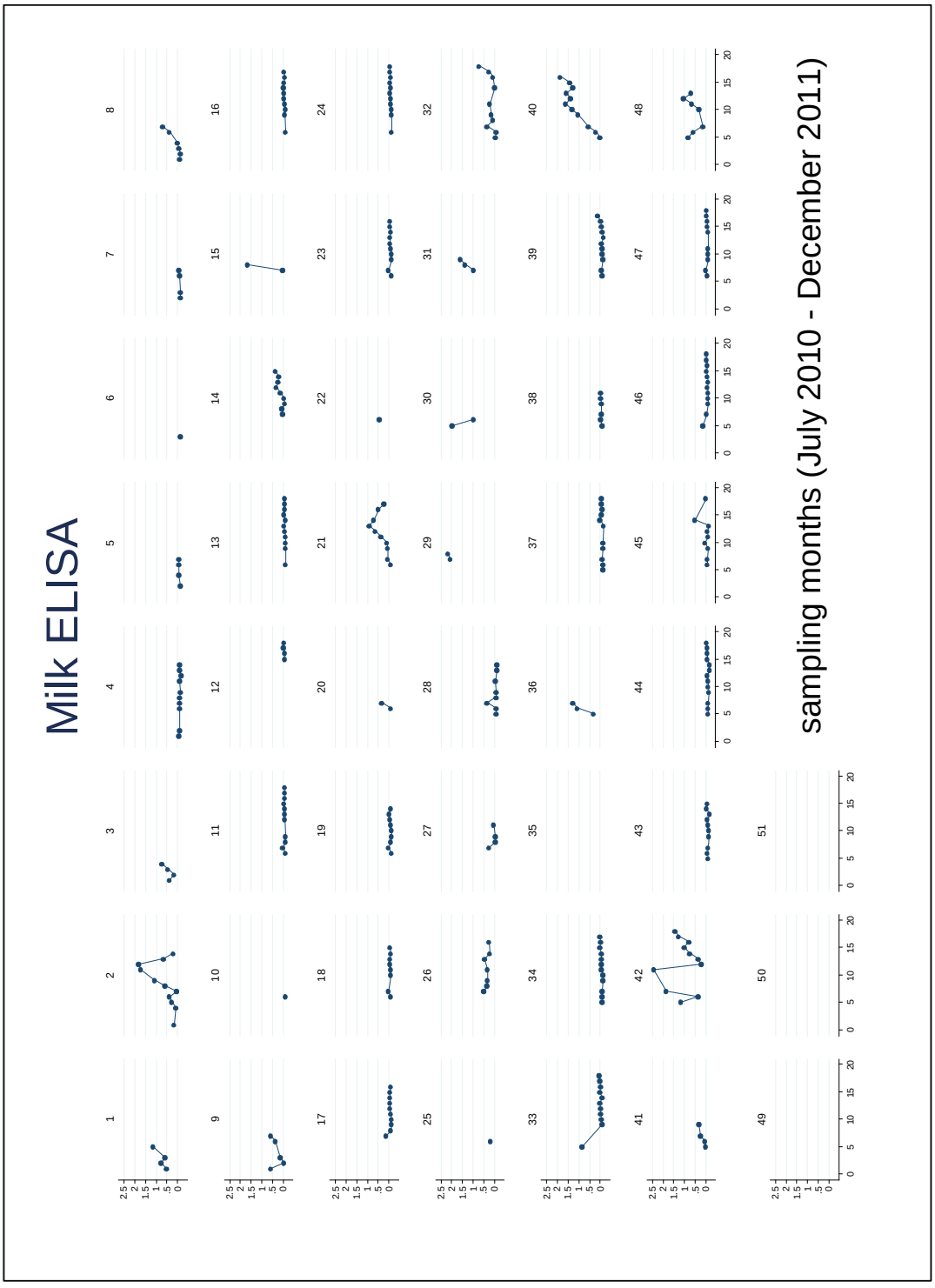
30
31


Fecal Direct Real-time PCR
sampling months (July 2010 - December 2011)


Milk Broth Culture
sampling months (July 2010 - December 2011)
high
moderate
pos/low
neg
0
5
10
15
20


Milk Solid Culture
sampling months (July 2010 - December 2011)
high
moderate
pos/low
neg
0
5
10
15
20


Milk Direct Real-time PCR
sampling months (July 2010 - December 2011)


Milk ELISA
sampling months (July 2010 - December 2011)